MOLECULAR GENETIC STUDIES IN HUMAN BREAST CANCER

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DECLARATION

In accordance with the regulations of the University of Edinburgh I declare that this thesis was composed by me and the work reported is my own except for those portions specified in the acknowledgements at the end of the text.

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SUMMARY

Paired tumour and blood samples from 100 patients with primary breast cancer, and 100 placental controls have been collected and in many instances lymphoblastoid cell lines have been established. DNA has been extracted from all these sources. Blood samples have been collected from all living members of a large family with a high incidence of breast cancer; DNA has been extracted and lymphoblastoid cell lines established.

The frequencies of the common and rare BamHI Harvey Ras alleles in breast cancer patients have been determined; there is no significant difference in their distribution when compared to controls from the same general population.

Allelic loss at the Harvey Ras locus was found in (chromosome 11p15.5) 13 out of 65 informative tumours and this loss is related to several clinico-pathoological indices, such as tumour size and oestrogen receptor status.

Five other regions on 11p have been fully examined in a panel of 19 tumour/lymphoblastoid cell line pairs. The data indicated that complex breakage and recombination events had occurred in at least a proportion of tumours, thus precluding identification of a shortest area of consistent deletion.

Several other areas of the genome have been studied to confirm that a high frequency of allelic loss is restricted to defined chromosomal regions.

Allelic loss of the locus defined by the minisatellite probe YNZ 22 on 17p 13.3 has been identified in 23 out of 38 informative tumours (61%). This loss is not related to any clinico-pathological parameters examined.

Taq 1 YNZ 22 genotypes for all members of a large kindred, containing 7 cases of breast cancer were identified. These genotypes were significantly linked to the trait of breast cancer susceptibility, with a calculated lod score of +1.8 at θ max - 0.

CONTENTS

INTRODUCTION

1 GENETICS OF BREAST CANCER

Historical Perspective

The identification of several "cancer-ridden" families stimulated a number of prominent 18th Century physicians to consider whether "there were any proofs of cancer being an hereditary disease?" (Shimkin 1957).

The remarkable pedigree of "Madam Z" was reported by the French surgeon, Paul Broca, in 1866. It is believed that the family described may indeed have been his wife's. He ascertained the cause of death in 38 individuals through 5 generations. Ten out of 24 women in that family died of breast cancer, and several more individuals died of other malignancies. Both Broca and his contemporary, Sir James Paget (Paget 1853; Lane-Clayton 1926) expressed concern that multiple instances of such a common disease might appear in a small number of families by coincidence, but using the available data on cancer mortality rates in the normal population, they concluded that a tendency to develop breast cancer could indeed be inherited.

Advances in statistics, epidemiology and genetics allowed more rigorous examination of these initial observations in the first half of the 20th Century. Several groups attempted to compare the mortality from breast cancer in a population of patients with one or more affected relatives and in a normal control population (Jacobson 1946; Woolf 1955; Anderson et al 1958; Kelsey 1978 and Lynch 1981). These studies highlighted the importance of pathological verification of malignancy, of assessing the incidence of malignancies other than breast cancer and of using large numbers of

families plus reliable data on cancer incidence and mortality in the general population. Overall they showed that there was a two-fold increase in breast cancer incidence in first degree female relatives of breast cancer patients. Under-reporting of disease by control subjects was recognised as a significant problem which remains hard to surmount.

Segregation Analysis

In some classical "genetic disorders", inspection of the pedigrees of a few affected families will reveal the mode of transmission (autosomal or sex-linked, dominant or recessive). However, in the vast majority of diseases, where the genetic component is less clearcut, a more comprehensive statistical analysis is required. Segregation analysis is the name given to the process of determining the probable mode of transmission of a trait, from an observed distribution of phenotypes in a pedigree or a number of pedigrees.

The procedure involves calculating how well the observed distribution of phenotypes fits various hypotheses and can thus establish that some of these hypotheses are very unlikely although it may not prove conclusively that a trait is transmitted genetically. The larger the families examined, the more affected individuals in each family and the larger the total number of families, the better will be the data and the more secure the final conclusions.

Several statistical advances, such as maximum likelihood scoring, the concept of multi-factorial inheritance, the "mixed model" and a sampling correction to allow for the manner in which the pedigrees have been ascertained and relatives added (Morton 1955, 1983; Falconer 1965, 1967; Morton and Maclean 1974; Elston and Sobel 1979; Lalouel and Morton 1981) have all been incorporated into

a segregation analysis, performed on 200 Danish families with breast cancer (Williams and Anderson 1984).

The observed distribution of breast cancer in these families was compatible with transmission of a single autosomal gene with dominant expression, the frequency of the abnormal (disease) allele being 0.7% and the penetrance varying with age. According to that model, by age 80, a female heterozygous for the abnormal allele would have a 57% chance of developing breast cancer. For cancer presenting before age 30, 88% of affected females would be carriers of the disease gene whereas for the total population, accepting all cases presenting up to age 80, only 13% of affected females would carry the gene.

Several similar analyses have been performed on another large breast cancer family and all agree that an autosomal dominant gene with incomplete penetrance is the most likely mode of transmission (Gardner and Stephens 1950; Hill et al 1978; Gardner 1980).

Hereditary Breast Cancer

On the basis of many published studies (Jacobsen 1946; Woolf 1955; Anderson et al 1958 and Kelsey 1979) it has been suggested that breast cancer can be divided into two sub-groups, familial and sporadic.

H T Lynch and colleagues (Lynch et al 1984) operationally defined "familial" breast cancer as the occurrence of two or more breast cancer affected relatives within the modified nuclear pedigree (inclusive of the proband). Families in which one or more of the following cardinal features were present were defined as having hereditary breast cancer.

1 Significantly early age of onset

2 Excess of bilaterality

3 Vertical transmission (mother-to-daughter)

4 Impaired survival when compared with sporadic forms

5 Excess of multiple primaries at various sites.

Applying these criteria, they estimated that 18% of breast cancer is familial, 5% of all breast cancer is hereditary and 11.5% of patients with breast cancer, diagnosed before the age of 50, have a hereditary form of the disease.

Anderson (1971, 1972) adopted a somewhat different approach, dividing his cases into sub-groups and identifying the groups in which the risk was increased beyond the two or three-fold level observed in the earlier studies (Jacobsen 1946; Kelsey 1979). The relative risk to first degree female relatives of patients with pre-menopausal breast cancer was 3.1, while no increase in risk was observed among relatives of post-menopausal patients. If the patient had bilateral breast cancer, the risk to first degree female relatives was increased five-fold. If both conditions applied (ie, the patient was pre-menopausal and had bilateral disease), the risk to first degree relatives was increased nine-fold. Furthermore, the relatives of patients with bilateral disease were at a nine times greater risk of developing bilateral disease themselves as compared to relatives of patients with unilateral disease.

The importance of age and of bilaterality have been confirmed in other studies (Bain et al 1980; Chaudray et al 1985) and so, despite one dissenting report from a large Swedish study in which the effects

of family history, age of onset and bilaterality were rather weak (Adami et al 1981), we are now in a position to identify a group of women who are at significantly higher risk of developing breast cancer than the general population, who are likely to be more aware of (and concerned about) their susceptibility to the disease (Kelly 1980), and who therefore require detailed and accurate counselling about that risk (Ottman et al 1983).

Several families have been reported in which breast cancer is associated with other primary neoplasms, such as endometrial carcinoma, (Lynch et al 1967), colonic carcinoma (Lynch et al 1973), ovarian carcinoma (Lynch et al 1978a), and leukaemia, brain tumours and sarcoma (Lynch et al 1978b). Multiple primaries can affect single individuals and/or different tumours occur among family members. These rare families may carry a different genetic lesion, influencing cell behaviour in a more fundamental way, than that occurring in families developing only breast cancer, but one advantage in studying them is that such a defect might be easier to detect if only because, with the opportunity for the gene to be expressed in a variety of forms, penetrance is expected to be relatively high.

The identification and study of both types of family is important in attempting to identify a gene for susceptibility to breast cancer and I shall return in a later section to consider the most informative method of analysing these families.

2 TUMOUR SUPPRESSOR GENES

The Two Hit Hypothesis

In 1971 A G Knudson (1971) published a hypothesis which provided a theoretical basis linking the molecular mechanisms underlying hereditary and sporadic cancers. He proposed that development of the malignant phenotype required two separate "hits", or mutations in sporadic tumours, but in hereditary tumours, the first "hit" is inherited in the germ line, and so only one somatic mutation is required. This hypothesis was based on a study of the childhood ocular tumour, retinoblastoma.

This tumour is usually recognised before 5 years of age, and has had a reasonably high cure rate for most of this century, permitting the early observation that some survivors produced affected offspring. In patients who have had bilateral tumours, about 50% of their offspring are affected and 50% are unaffected, suggesting such patients bear a "retinoblastoma mutation" which is transmitted in dominant Mendelian fashion. Most patients with a unilateral tumour, produce unaffected offspring, although 10-15% are affected. Most of the latter develop bilateral tumours.

Knudson calculated that 60-75% of gene carriers develop bilateral tumours, 25-40% develop unilateral tumours and between 1 and 10% do not develop the disease, although the trait is transmitted to their offspring, suggesting the number of tumours developing in a gene carrier fits a Poisson distribution. Pathological data confirmed that gene carriers develop a mean number of 3-4 tumours.

Thirty-five to 40% of total retinoblastoma cases appear to be "hereditary" and the incidence of these is 5 per 100,000 children. Therefore the probability that a non-carrier will develop the disease

is 3 per 100,000. As the mean number of tumours per gene-carrier is 3, the risk for one tumour in gene-carriers relative to non-carriers is 100,000:1 (Knudson 1978).

There are 10^6 cells in the normal retina, so even in gene-carriers, it is a very rare cell that becomes malignant, strongly suggesting a second event is involved, following a Poisson distribution, and therefore likely to be random. Knudson calculated that the frequency of this second "hit" was roughly equal to the frequency of the first "hit" acquired in non-hereditary tumours and likely to be a mutational event (Knudson 1971). This mathematical treatment could not of course cast light on whether the second event in non-hereditary tumours is the same as the first event in hereditary tumours.

Localisation of the Retinoblastoma Gene

In some families with retinoblastoma, the disease is inherited along with a visible deletion in the long arm (q) of one of the chromosome 13 pair. These deletions are of variable length but all include one particular band - 13q 14 (Knudson 1976; Yunis 1978).

The gene coding for the polymorphic enzyme esterase D, was shown to be located at 13q 14 (Chen 1974; Ward 1984). The serum levels of esterase D are reduced to 50% of normal in those with a visible deletion, (and in several without visible deletions), suggesting that even submicroscopic deletions include both the esterase D gene and the retinoblastoma gene (Sparkes 1980). There are two allelic forms of esterase D in the normal population, EsD 1 and EsD 2, and in informative pedigrees one allele segregated with the disease. (Connolly 1983; Sparkes 1983). This is good evidence that the same

genetic site i.e. 13q 14 is involved in all hereditary forms of retinoblastoma, irrespective of whether a visible deletion is present or not.

Cytogenetic analysis was possible in about 20% of non-hereditary tumours, and all showed absence of, or a deletion on, one of the No 13 chromosomes - all involving 13q 14 (Balaban 1982; Benedict 1983a; Gardner 1982), suggesting that the same locus is involved in both hereditary and non-hereditary forms.

Expression of the Retinoblastoma Gene

Three classes of events could lead to loss of the normal allele at the retinoblastoma locus (Knudson 1978).

1 Submicroscopic mutation (designated $13q^{rb}$, the normal allele being $13q^{+}$.

2 Deletion ($13q^{-}$).

3 Chromosomal loss (13-).

If expression of the retinoblastoma gene is recessive, and the second event involves the development of homozygosity to allow expression, then 6 tumour genotypes can be imagined, $13q^{rb}/13q^{rb}$, $13q^{rb}/13q^{-}$, $13q^{rb}/13^{-}$, $13q^{-}/13q^{-}$, $13q^{-}/13^{-}$, $13^{-}/13^{-}$. The last genotype would probably be fatal as would the fourth and fifth if enough genes were lost; however, the first three genotypes might be compatible with tumour cell survival.

The first event, a mutation, is to $13q^{rb}$, and the second event is then another mutation in the first case, a deletion in the second, and complete chromosomal loss by non-disjunction in the third. The first genotype could also be caused by somatic recombination, whereby a heterozygous cell, $13q^{rb}/13q^{+}$, produces two homozygous cells, one normal, $13q^{+}/13q^{+}$, and the other tumourigenic, $13q^{rb}/13q^{rb}$.

The first evidence that the retinoblastoma gene was rececessive came from studying a hereditary case with no visible deletion in the leucocytes, in which the serum esterase D level was 50% of normal, suggesting a submicroscopic deletion of the retinoblastoma and esterase D genes in one copy of chromosome 13. The tumour cells had only one copy of chromosome 13, and had no esterase D activity, suggesting that the tumour genotype was therefore $13q^{-}/13^{-}$, and the tumour had lost the normal chromosome 13 (Benedict 1983b; Murphree 1984).

The identification of several highly polymorphic DNA segments on the long arm of chromosome 13 (Cavenee 1983, 1985), has provided further evidence that the retinoblastoma gene is recessive. Some tumours in heterozygous individuals have lost one allele, indicating that a portion of one copy of chromosome 13 has been lost, i.e. $13q^{rb}/13q^{-}$, while in some tumours all heterozygous markers have been reduced to apparent homozygosity (Dryja 1984), indicating probable loss of one chromosome and the acquisition of a second copy of the other chromosome by non-disjunction. A few tumours show no loss of heterozygosity, with no esterase D deficiency and are presumably $13q^{rb}/13q^{rb}$, while others show no loss of heterozygosity but reduction of esterase D activity, suggesting a submicroscopic deletion involving the retinoblastoma gene and the esterase D gene, but none of the other markers (Dryja 1984).

These findings in retinoblastoma indicate that the retinoblastoma gene is recessive at a cellular level, and that a second event is required to allow its expression. In hereditary retinoblastoma, every one of the 10^6 cells in the retina have inherited the first "hit" and so the chances of at least one cell acquiring the

second "hit" are so high that the trait (i.e. development of at least one tumour) appears to be transmitted as a Mendelian dominant characteristic. In some ways the term "hereditary retinoblastoma" is misleading, as it is the predisposition to develop a tumour which is inherited (Knudson 1985).

The normal copy of the retinoblastoma gene ($13q^{+}$) appears to suppress the activity of the mutated gene ($13q^{rb}$) and therefore acts as a tumour suppressor gene. Similar experimental findings have been reported in Wilm's tumour (nephroblastoma), hepatoblastoma, and rhabdomyosarcoma (Koufos 1984, 1985; Orkin 1984; Reeve 1984; Fearon 1984 and Rogler 1985), but the gene implicated in these tumours lies on the short arm of chromosome 11 (11p). It is likely that a number of different tumour suppressor genes are scattered throughout the genome (Lancet 1988). It is therefore logical to examine DNA from both hereditary and non-hereditary tumours to attempt to identify sequences which are consistently lost in the tumour tissue, as these sequences might well be new tumour suppressor genes.

Another term coined for tumour suppressor genes is anti-oncogenes (Knudson 1985; Green 1985; Friend 1988). They appear to act in a recessive fashion at the cellular level in contra-distinction to the larger number of oncogenes identified, which act in a dominant fashion at the cellular level, and which will be discussed in detail in the next section.

3 ONCOGENES

Experimental approaches

The identification of DNA sequences capable of producing a malignant cellular phenotype in a dominant fashion, i.e. without loss of the normal homologue, has resulted from three main experimental approaches; investigation of tumour-producing viruses, transfection of human tumorigenic DNA into immortalised cell lines and visual chromosome analysis.

The first clues came from an understanding of the structures of oncogenic retroviruses. The genetic material of these viruses is RNA but once inside an infected cell, the RNA is copied into DNA which then integrates into the chromosomal DNA of the host. Later it may be transcribed and translated by the host cell's replication system, unleashing a second generation of virus. These viruses can be divided into two broad groups, depending on the rapidity with which they produce tumours. Slowly transforming viruses contain three genes; "gag", which codes for specific antigens mainly located at the core of the virus, "pol", which codes for reverse transcriptase and "env", which codes for the envelope protein. The acutely transforming viruses contain another gene termed an "oncogene", specific to each virus and directly responsible for induction of malignancy in infected cells (Bister 1982; Yoshinaka 1985).

Analysis of the nucleotide sequences of retroviral oncogenes revealed that they were very similar to sequences ("proto-oncogenes") found in the genome of higher organisms, including man (Stehelin 1976; Bishop 1981). It is virtually certain that the acutely

transforming viruses arose by recombination between non-oncogenic or slowly transforming viruses and the cellular proto-oncogenes. At least 22 viral oncogenes have been identified so far (Bishop 1983).

The second productive experimental approach has been direct DNA transfection/transformation. DNA from human tumours or tumour cell lines is precipitated onto the surface of the cells in culture using calcium phosphate, or by electroporation (Steel 1984). A small proportion of the DNA enters the cells and an even smaller fraction becomes integrated into the genome in a random fashion. Cells "transformed" by this technique exhibit uncontrolled proliferation and form distinct colonies on agar plates, which can be isolated and cloned up (Shih 1982). When injected into immunologically-compromised animals, such as nude or neonatally thymectomised and irradiated mice, these transformed cells produce tumours, suggesting that a segment of DNA responsible for producing the original human tumour, has been integrated into the cell's genome (Krontiris 1981; Porteous 1986). These oncogenes can then be identified by comparing extracted DNA from the transformed cells and from the original cell line.

Eleven new cellular oncogenes, without viral counterparts have been identified by this technique (Bishop 1983). Among the few oncogenes identified by both transfection and viral studies are members of the ras gene family which will be considered more fully later.

One of the main limitations of DNA transfection studies is that the target cells used, usually 3T3 or C127 mouse fibroblasts, though subject to some normal growth controls, are immortal. They

therefore represent an intermediate stage between the normal and full-blown malignant states. Co-transfection of at least two different oncogenes, for example, Ha-ras and C-Myc is generally required to achieve tumorigenic transformation of normal cells (Land 1983).

The molecular mechanisms underlying the process of immortalising a normal cell are very poorly understood, but it is likely that transfection studies are capable of detecting only a restricted range of oncogenic events. Transfection of the retinoblastoma gene (13_{rb}) into an immortalised cell, for example, would not produce a tumorigenic cell, as the normal homologue ($13q_{+}$) would still be present. However, transfection of the normal homologue (13q+) into a tumorigenic cell line containing only the retinoblastoma gene ($13q^{rb}$), would be expected to convert that tumorigenic cell line into a non-malignant line. It is therefore clear that transfection studies can be a very powerful tool in elucidating the molecular mechanisms underlying malignant development. If other studies have already yielded information as to the structure and/or location of the gene involved.

The third fruitful experimental approach is high resolution chromosome banding of malignant cells. Although many of the steps involved in malignant change are submicroscopic, several consistent visible rearrangements have been reported (Rowley 1982; Mitelman 1984). The easiest neoplastic cells to examine in this fashion come from haematological malignancies, and the first translocations identified were the 9:22 translocation in chronic myelogenous leukaemia and the 8:14 translocation in Burkitt's lymphoma (Klein 1983; Heisterkamp 1983; Groffen 1984). Both these translocations

involve previously identified oncogenes, c-abl and c-myc respectively, but three new oncogenes, bcl-1, bcl-2 and tcl-1 have been identified by similar studies in B-cell leukaemia, B-cell lymphoma and T-cell leukaemia (Tsujimoto 1984, 1985; Croce 1985).

Combining these three experimental approaches, around 45 sequences in the human genome have so far been classed as proto-oncogenes (Bishop 1983; Bloomfield 1988), although this number may well increase.

Methods of activation

Proto-oncgenes are activated to become tumorigenic by a variety of changes in either their structure or their regulatory elements.

1 Point mutation.

The three members of the ras gene family, Harvey ras, Kirsten ras and N-ras, were identified by transfection experiments from a bladder carcinoma cell line, a lung carcinoma cell line and a neuroblastoma cell line (Der 1982; Shimizu 1983a, Fasano 1983; Shimizu 1983b). c-Harvey ras shares extensive homology with the oncgene of the Harvey murine sarcoma virus (McBride 1982; Schwartz 1983) and c-Kirsten ras is homologous to the Kirsten sarcoma virus (Chang 1982). These three genes are very similar (Ellis 1981), although dispersed through the human genome, Harvey ras on 11p (McBride 1982; de Martinville 1983), Kirsten ras on 12p (Jhanwar 1983), and N-ras on 1p (Hall 1983), encode three very similar proteins of 21,000 molecular weight, known as p21 (Capon 1983; Taparowsky 1983; Lacal 1984).

Sequencing the proto-oncgene and the activated oncgene has revealed that, in each case, a single point mutation activates the proto-oncogene producing an abnormal p21. In Harvey ras this mutation is in codon 12 or codon 61 (Reddy 1982; Goldfarb 1982).

Injection of the abnormal Harvey ras p21 into immortalised cells transforms them into tumorigenic cells, indicating that the single amino acid substitution in the protein product is enough to confer the malignant phenotype (Taparowsky 1982).

2 Increased transcription

Abnormally high levels of the normal p21, induced by increasing the rate of transcription or the number of copies of the Harvey ras proto-oncogene and thus the rate of translation of the normal protein product, will also transform 3T3 cells in culture, thus a point mutation is not essential (Parada 1982; Pulciani 1985).

Another proto-oncogene, c-erb B2, homologous to the avian erythroblastosis virus located on 17q (Bister 1979; Jansson 1983) is amplified in a variety of tumours and tumour cell lines (Ullrich 1984; Semba 1985; Fukushige 1986).

3 Production of a hybrid m-RNA

In chronic myeloid leukaemia, translocation between portions of chromosomes 9 and 22 results in the juxtaposition of the oncgene c-abl and the bcr (breakpoint cluster region), resulting in the transcription of a hybrid m-RNA molecule containing some coding exons from bcr and all the coding exons except the first, from c-abl, which is translated into an abl protein with a novel amino terminus derived from the bcr gene (Gale 1984; Heisterkamp 1985). The abl product has protein kinase activity and the fusion protein has altered enzyme kinetics.

4 Dysregulation

Translocation of the oncgene, c-myc from chromosome 8 to chromosome 14 seen in Burkitt's lymphoma results in dysregulation of c-myc leading to a continued expression of the normal gene (Klein 1981;

Dalla-Favera 1982; Leder 1983; Taub 1984) whereas expression is usually terminated as the cell differentiates (Curran 1984; Gonda 1984).

It is clear from the preceding sections that a large number of oncogenes have been identified, and progress is being made in understanding the various mechanisms by which a proto-oncogene becomes activated, to cause a wide variety of human tumours. Those oncgenes that have been implicated in human breast cancer, and their possible contribution to the aetiology of the disease will be examined in the next section.

4 ONCOGENES IMPLICATED IN HUMAN BREAST CANCER

c-myc

Amplification of the proto-oncgene c-myc, on chromosome 8, has been reported in a variety of carcinoma cell lines (Little 1983). Between 20 and 30% of primary breast tumours show amplification of this gene (Escot 1986; Varley 1987), but elevated levels of expression have been reported in up to 80% of breast tumours (Kozbar 1984; Lee 1984). Thus amplification is not essential for high levels of expression, although in most cases, amplification leads to high levels of c-myc m-RNA.

Amplification of c-myc was significantly more frequent in patients older than 50 years of age at presentation in one study (Escot 1986), but was not linked to oestrogen or progesterone receptor status, tumour grade or axillary node metastasis at presentation. Another study (Varley 1987) found no correlation between amplified c-myc and oestrogen receptor status, menopausal status, or stage of the disease; but there was a significant correlation between c-myc amplification and a poor prognosis as measured by early recurrence of disease or death.

Although these studies examined fairly small numbers of patients, they suggest that amplification of c-myc may well be a late event in progression of a breast tumour. This suggestion ties in with the finding that another member of the myc family N-myc is amplified in Grade 2 and 3 neuroblastomas, but not in Grade 1 neuroblastomas (Brodeur 1984).

There have been two independent reports of a rearrangement of the c-myc locus in breast tumour DNA (Escot 1986; Varley 1987). This rearrangement has been extensively mapped (Varley 1987) but the

very low frequency with which it occurs, twice in a total of 162 tumours studied in the two reports, suggests that this event is unlikely to be of great significance in the development of breast cancer.

erb B2

Transfection studies with DNA from chemically-induced rat neuroglioblastomas identified an oncgene, named neu. Two other groups independently isolated a similar oncogene and named it HER-2 and c-erb B2. Subsequent analysis has revealed that all three genes are the same (Shih 1981; Coussens 1985; Semba 1985). This gene codes for a protein similar to, but distinct from, epidermal growth factor (Schechter 1984; Coussens 1985) and is amplified 5- to 10-fold in a mammary carcinoma cell line (King 1985). It was then reported (Slamon 1987) that 18% of primary breast tumours have amplification of erb B2 from 2- to 10-fold, and the only significant clinical pathological correlation established was with metastatic lymph node invasion. A second study identified erb B2 amplification in 40% of patients who were lymph node-positive at presentation, and in this subgroup of patients there was a strong correlation between erb B2 amplification and disease-free interval and survival, with erb B2 amplification being a poor prognostic factor (Slamon 1987).

One group, which looked at both c-myc and erb B2 amplification reported that both genes are not amplified in the same tumour, while the prognosis for patients with amplification of one of them is essentially the same, irrespective of which gene is amplified, and significantly poorer than for patients in whom neither gene is amplified. This suggests that c-myc and erb B2 interact in some way in the normal cell to regulate cell proliferation and that disturb-

ances of regulation of either sequence in malignant breast epithelium may result in the same clinical phenotype, i.e. poor short-term prognosis (Varley 1987).

int-2

Mouse mammary tumour virus has been identified as a biological agent associated with a high incidence of breast cancer in certain inbred strains of mice (Moore 1974; Varmus 1982). No transforming oncogene has been identified in the virus and a high percentage of mammary epithelial cells may be productively infected yet still maintain a normal phenotype. Tumours are rare, relative to the total numbers of infected cells, and usually appear after a delay of several months following initial infection. This pattern of tumorigenicity suggested the possibility of insertional mutagenesis with perturbation of expression of endogenous host proto-oncgenes (Varmus 1982; Nusse 1984). In most MMTV-induced mammary cancers, proviral insertion has been detected in one or other of two distinct loci designated int-1 and int-2 (Nusse 1984; Dickson 1984). Genes homologous to mouse int-2 have been identified in other mammalian species including man (Casey 1986) and the human int-2 gene has been mapped to 11q13 (Casey 1986).

Amplification of int-2 has been reported in 4 out of 46 primary breast tumours, all of which had metastised to regional lymph nodes at presentation and all four behaved aggressively, progressing within 3 to 8 months despite intensive chemotherapy (Zhou 1988).

All the studies quoted in this section, and most strikingly the last study, suggest that amplification of oncogene sequences occurs in only a proportion of tumours and tends to generate aggressive

malignant behaviour. It is therefore likely to occur late in the lifetime of a neoplasm and to be of more importance in the progression of the disease rather than its aetiology.

Harvey ras

An activating point mutation in codon 12 of the Harvey ras gene has been identified in 80% of rat mammary carcinomas induced by nitroso-methyl urea (Sukumar 1983), and in a human cell line derived from a rare mammary carcino-sarcoma (Kraus 1984) but has not been identified in primary breast tumours (Theillet 1986). However, several groups have reported high levels of the normal protein product p21 (Ohuchi 1986; Horan-Hand 1984, 1986; Whittaker 1986) and Harvey ras m-RNA (Spandidos 1984) in up to 70% of human primary breast tumours. One report correlates high levels with p21 with larger tumours, metastatic axillary lymph node involvement at presentation, and a shorter disease-free interval (Clair 1987), suggesting that tumours with a high level of p21 exhibit more aggressive behaviour, and that measurement of p21 in breast tumours might be a useful prognostic indicator.

p53

p53 is a very interesting gene, which has perhaps received less attention than it deserves in relation to human malignant disease. The product, a protein of molecular weight 53,000 is a DNA binding nuclear protein found in a wide variety of transformed cell systems.

Several studies have indicated that the expression of the p53 gene is associated with cell cycling and cell proliferation (Reich 1984; Mercer 1985) suggesting that it normally functions in the control of cell growth.

The gene was first identified by immunological techniques (Lane 1979; DeLeo 1979), but there was a lag of 5 years before demonstration of its oncogenic potential as an immortalising gene (Jenkins 1984). Transfection of a codon 12-mutated ras gene will only transform an immortal cell into a tumorigenic cell, but cotransfection with p53 will transform rat embryo fibroblasts into fully malignant cells (Eliyahu 1984; Parada 1984).

However, in this early work it was essential to have p53 expression markedly enhanced by powerful viral promotors (Eliyahu 1984). Jenkins (1985) transfected p53 genes mutated *in vitro* and linked to a relatively weak promotor into rat embryo fibroblasts, and was then able to induce transformed foci by secondary transfection with an activated ras gene. These mutated p53 genes coded for mutant proteins which were much more stable in the cell, and it therefore seemed that p53 could only act as an immortalising gene, if the cellular protein levels were abnormally high, either through increased transcription or reduced protein breakdown. However, transfection of p53 from normal mouse liver has recently been reported to immortalise rat embryo fibroblasts (Rovinski 1988). Whether this gene has, in fact, mutated *in vivo* remains an open question (Oncogene 1988).

Elevated levels of the p53 protein were initially reported in 24% of breast cancers, particularly high-grade tumours (Crawford 1984). A subsequent report confirms this, finding high levels of the protein p53 in 15% of primary breast cancers, significantly correlating with histological Grade III tumours, with low levels of oestrogen receptor protein, and high levels of epidermal growth factor receptor (EGFR) protein (Cattoretti 1988).

Low levels of oestrogen receptor protein (Moore 1983; Williams 1987) and high levels of EGFR protein (Sainsbury 1985, 1987) are both accepted as poor prognostic indicators in breast cancer.

This gene's ability to cooperate with mutated ras in transforming mortal rat fibroblasts, its elevated expression in the percentage of apparently aggressive human breast cancers, and its localisation in the human genome to the short arm of chromosome 17 (Isobe 1986; McBride 1986; Miller 1986) suggests that examining chromosome 17 in breast cancer patients might well be fruitful.

Having discussed the major oncogenes so far implicated in breast cancer, we will now turn to examine the various strategies worth pursuing in the search for those genes determining susceptibility to breast cancer.

4 THE SEARCH FOR BREAST CANCER "SUSCEPTIBILITY" GENES

Identifying tumour suppressor genes

In the preceding section I have argued that almost all the reported alterations in known oncogene sequences in human breast cancer are correlated with various parameters of aggressive tumour behaviour, and therefore likely to be late events in the tumour's lifespan. However, the inference from the findings in retinoblastoma would justify a comparison of DNA from both hereditary and non-hereditary "early" tumours with DNA from somatic tissue, in order to try and identify sequences which have been lost in these "early" tumours. This approach has been greatly facilitated by the recent identification of a large number of highly polymorphic DNA sequences (White 1985; Barinaga 1987). Because they are very polymorphic, a high percentage of individuals are constitutionally heterozygous, and it is therefore clearer if the tumour DNA has lost an allele.

This rather theoretical concept has recently gained strong experimental support from studies on familial polyposis coli and colorectal carcinoma.

Following a case report of an interstitial deletion on 5q in a mentally retarded patient with features suggesting familial polyposis coli, but without appropriate family history (Herrera 1986) a highly polymorphic "minisatellite" probe, mapping to 5q34 (Wong 1987), revealed loss of a portion of 5q in up to 40% of sporadic colorectal carcinomas (Solomon 1987). At the same time formal linkage analysis using several other polymorphic 5q probes localised the gene for familial polyposis coli to 5q 21-22 (Bodmer 1987; Leppert 1987). I shall return to the theoretical basis and applications of linkage analysis in the third part of this section.

Reduction of a heterozygous marker to homozygosity in tumour DNA has been reported in a variety of other cancers. In Wilm's tumour (nephroblastoma), hepatoblastoma and rhabdomyosarcoma a consistent deletion on 11p has been reported (Koufos 1985).

Two groups have reported loss of a Harvey ras allele (mapping to the tip of 11p) in a significant proportion of breast cancers (Theillet 1986; Yokota 1986), and one of these groups has reported that the most frequent loss of sequences occurs between the beta-globin and parathyroid hormone loci on 11p (Ali 1987).

Loss of heterozygosity in a number of chromosomal locations has also been reported in lung cancer (3p) (Kok 1987), renal cell carcinoma (3p) (Zbar 1987), transitional cell bladder carcinoma (11p) (Fearon 1985), osteosarcoma (13q) (Dryja 1986), uveal melanoma (2) (Mukai 1986), acoustic neuroma/meningeoma (22) (Seizinger 1986), and adrenal adenoma (11p) (Hayward 1988a).

The situation has been further complicated by reports of loss of heterozygosity on 17p in 80% of colorectal carcinomas (Fearon 1987), and on 13q in 60% of breast carcinomas (Lundberg 1987).

It is quite possible that these reports are compatible with each other (Lancet 1988), as it is widely accepted that carcinogenesis is a multi-step process and loss of two or more tumour suppressor sequences might be essential before a cell can express the fully-blown malignant phenotype.

Unusual allele distribution

The second potentially useful strategy is to examine different populations for an unusual distribution of alleles of a particular marker. The well-known finding that both gastric carcinoma and

pernicious anaemia occur more often in individuals of blood group A is a good example, but due to the large number of unaffected individuals of blood group A, it is of no help in screening for gastric carcinoma.

On the other hand 90% of patients with ankylosing spondylitis have the histocompatibility antigen HLA B27, and I shall return to the importance of this in screening when I examine linkage analysis (Tiwari 1985).

It is possible to identify several polymorphic forms of the DNA sequence bearing the Harvey ras proto-oncogene in the normal population, by DNA digestion and Southern blotting (see Section 5). Using this technique in 1985, Krontiris reported his findings in a group of patients with a variety of haemopoietic and solid malignancies including a small number of breast cancers. In the normal population, he identified four common c-Harvey ras alleles and a number of rare alleles, and in cancer patients he found a significantly higher frequency of rare alleles. He suggested that possessing a rare Harvey ras allele indicated that the individual would be more susceptible to development of cancer. Several groups have now repeated this work and in general it has not been substantiated.

Heighway (1986) reported a preponderance of one of the common alleles in patients with non-small cell lung carcinoma but no increase in frequency of rare alleles in either small cell lung cancer or non-small cell lung cancer. Similar studies have reported negative results in myelodysplasia (Thein 1986), colonic adenocarcinoma (Ceccherini-Nelli 1987), sporadic and familial melanoma (Gerhard 1987), and Wilm's tumour (Hayward 1988b), although an increased frequency of rare Harvey ras alleles has been reported in

bladder carcinoma (Hayward 1988b), and a large series of patients with breast cancer (Lidereau 1986), in which 104 breast cancer patients and 56 controls were examined. 58.6% of the 208 alleles in breast cancer patients were "common" compared to 91% of the 112 control alleles. Lidereau and colleagues reported no correlation between presence of a rare allele and any clinico-pathological factors. In all the studies quoted, the frequency of each of the four common alleles in the control populations are very similar, although Lidereau's estimation of the molecular weight of these common alleles differs by up to 800 base pairs from that of Krontiris. These differences are ascribed to experimental error.

The differences in molecular weight between the rare and common alleles identified is never more than 300 base pairs and there appear to be no similarities between the rare alleles identified in the separate studies of Kronitiris and Lidereau.

Whether there is an increased frequency of rare Harvey ras alleles in any form of cancer remains an open question, which will only be answered by more meticulous studies with larger numbers of patients.

Lidereau's group has also reported an unusual allele of the c-mos proto-oncgene on 8q 22 in 6 out of 75 breast cancer patients (Lidereau 1985) but the significance of this is obscure in the absence of a formal linkage analysis.

Linkage analysis

The most practical approach to locating the gene for susceptibility to breast cancer is by the technique of linkage analysis, based on the segregation of defined genetic markers in affected and

unaffected family members (Yates 1986). The human genome is composed of genes arranged in a linear fashion along the 23 pairs of chromosomes. Genes which are close together on the same chromosome tend to be transmitted together - i.e. to segregate non-independently. Genes on different chromosomes segregate independently so that every possible combination of alleles appears with equal frequency in the gametes as illustrated by Figure 1. The disease gene is D (normal allele-d) and the marker gene alleles are represented by Tt.

Figure 1

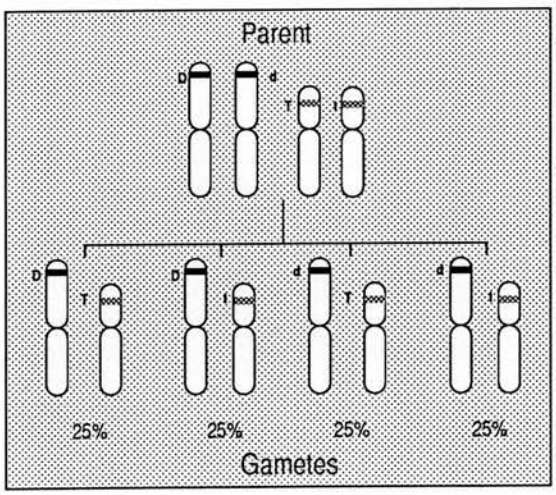


Fig. 1. Independent assortment of alleles at meiosis for a disease locus (alleles D and d) and a marker locus (alleles T and t) on different chromosomes

If the disease gene and the marker gene are physically very close together, they will be transmitted together, so that the gametes formed are either DT or dt as shown in Figure 2.

Figure 2

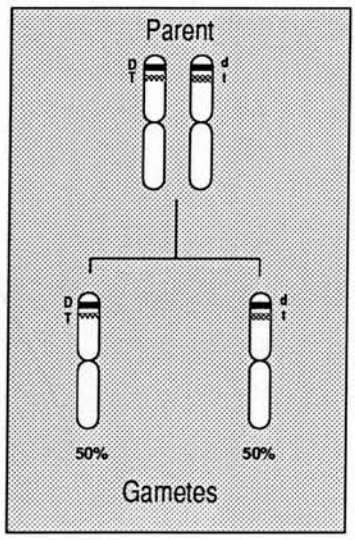


Fig. 2. Absence of independent assortment of alleles at meiosis for disease and marker loci very close together on the same chromosome (tight linkage).

This depature from independent segregation is termed "linkage", with D and T being very tightly linked in the examples shown. If D and T are slightly further apart on the chromosome, the two genes may well be transmitted together but because they can segregate by crossing over and recombination at meiosis, a few gametes with genotypes Dt or dT will appear, as illustrated in Figure 3.

Figure 3

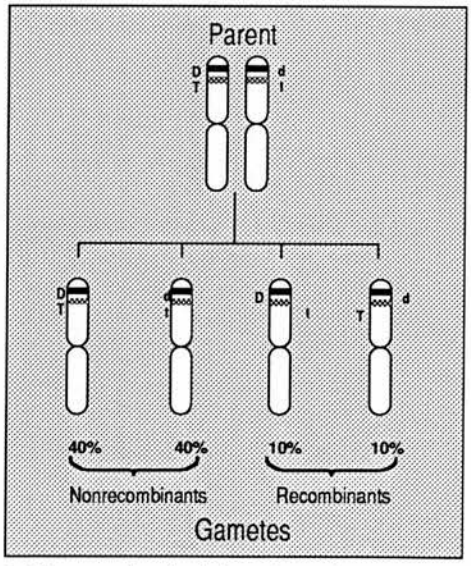


Fig. 3. Disease and marker loci nearby on the same chromosome showing linkage. The recombination fraction is 20%.

These gametes are known as recombinants and the proportion of recombinant gametes in the total pool is the recombination fraction.

The further apart the two genes are, the more likely it is that recombination will occur, until eventually the two genes will appear to be segregating independently as all four possible genotypes will be represented, giving a recombinant fraction of 0.5 as illustrated in Figure 4.

Figure 4

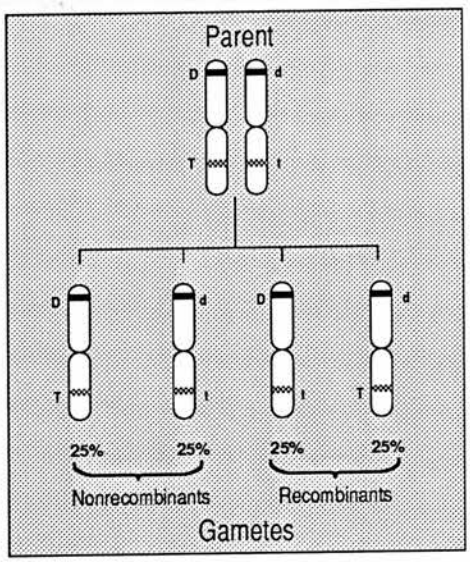


Fig. 4. Disease and marker loci far apart on the same chromosome mimic independent assortment and linkage cannot be detected. The recombination fraction is 50%.

The markers used in linkage analysis must be polymorphic, (i.e. more than one allelic form found in the population), and ideally the chromosomal location should be known. After identifying which allelic forms of the marker are present in every individual (both affected and unaffected) in a family, it is possible to calculate, A: the probability of the observed distribution occurring by chance if there is no linkage between the marker gene and the disease and, B: the probabilities of such a pattern appearing if there is linkage at

different recombination fractions. The logarithm of B/A known as the "lod" (logarithm of odds) score, is calculated for various recombination fractions. To obtain statistical significance it is usually necessary to combine data from several families and the lod scores from each family for each recombination fraction can be added together. A lod score greater than +3 is usually taken as demonstrating significant linkage but obviously the more families examined, the greater the confidence one can place in the lod score.

King, Go and colleagues (King 1983; Go 1983) used segregation analysis to identify 10 families showing genetic transmission of a breast cancer trait and performed linkage analysis using 21 independent polymorphic markers. They suggested that in 7 families there was significant linkage between an autosomal dominant gene for susceptibility to breast cancer and the enzyme glutamate-pyruvate transaminase (GPT), the gene for which is located on chromosome 16 (Wijnen 1982; McKusick 1982). Go (1983) reported a lod score of +1.95, at a recombination fraction of zero and accepted that such a result should be viewed with caution. When a larger number of families was examined it was conclusively shown that there was no linkage between GPT and susceptibility to breast cancer (McLellan 1984).

Confusion sometimes arises between the terms "linkage" and "association" in the context of genetics. As explained, linkage between two DNA sequences means that they are physically close together in the genome hence if <u>one</u> is polymorphic, so that inheritance of the different alleles can be traced within a family, it can be used to "tag" the other sequence. It does not follow that the <u>same</u> allelic forms of the two sequences are linked in all cases.

For example, in the case illustrated in Figure 2, a single chromosome carries D and T in tight linkage, so that "T" would serve as a marker for "D" in this family. In another family however, the relevant chromosome might carry the alleles D and t, in which case the marker for "D" would be "t". In other words, there is no functional relationship between the gene with alleles Dd and that with alleles Tt. The important practical consequence is that even if strong linkage is established between these genes, there is no purpose in screening a population, say for carriers of the "t" allele, since one could not predict which of the individuals so identified would also carry "D". That type of prediction is valid only <u>within</u> each family.

"Association" is quite a different matter. The term implies that a particular <u>allele</u> of a given gene is over-repeated among the total population of patients with a particular genetic disorder. There are, for example, over 100 diseases associated with individual alleles of the major histocompatability system (Tiwari 1985). The associations between HLA-B 27 and ankylosing spondylitis or DR 4 and rheumatoid arthritis are well-known instances. These associations hold across family boundaries (although, interestingly, they may not apply in all racial groups) and can therefore be useful in population screening. Association in this sense may come about because the disease arose through a single mutational event affecting a gene so close to the "marker" sequence, that the disease and marker alleles have never become separated through the succeeding generations. This so-called "founder effect" implies that all the affected individuals in a population are actually related so they may not be aware of it. An equally likely mechanism however, is a <u>causal</u>

relationship between the marker allele and the disease itself. In other words, individuals with the HLA-type B 27 are at risk from ankylosing spondylitis, not because an "ank-spond" gene lies close to the HLA complex on the short arm of chromosome 6, but because the B 27 gene product is actually involved in the aetiology of the disease.

Returning to the problem of genetic susceptibility to breast cancer, the search for linkage in the strict sense, simply means extension of the approach used by King, Go and their colleagues, namely the analysis of large numbers of randomly chosen, polymorphic markers (Nakamura 1987) in families with multiple cases of disease occurring before the menopause. An alternative approach would be to try to identify "candidate" genes suspected, for one reason or another, of possible involvement in breast cancer. DNA sequences which have been lost in tumour tissue, and represent putative tumour suppressor genes, are obvious candidates for this approach. Until recently, the shortage of useful genetic polymorphisms has restricted both of these approaches, but the situation has been transformed by the discovery of DNA restriction fragment-length polymorphisms as discussed in the next section.

5 SOUTHERN BLOTTING AND RESTRICTION FRAGMENT-LENGTH POLYMORPHISMS

Native DNA is a double-stranded helix, and because adenine must be opposite thymidine, and guanine opposite cytosine, a single strand of DNA uniquely defines its complementary strand of DNA or RNA.

Under appropriate conditions of pH, temperature and ionic strength, single-stranded DNA or RNA fragments will hybridise with complementary single strands of DNA. The stability of the double-stranded complex ("hybrid") depends upon the degree of complementarity between the two nucleic acid strands. By increasing the pH or temperature or altering the ionic strength, hybridisation conditions can be made more stringent until only strands that are perfectly matched at every base pair will remain as hybrids. This property is exploited in the technique of gene probing.

After extraction from the lysed cells and purification, as described in Materials and Methods section, DNA is digested with a restriction enzyme. Many different restriction enzymes, isolated from bacteria and fungi, are now commercially available. Each enzyme recognizes a specific base sequence in double-stranded DNA and cuts both strands wherever that sequence occurs (Roberts 1983). The fragments thus produced can be separated by electrophoresis in an agarose gel. The shorter fragments will move faster through the gel and so travel further away from the origin in a given time than large, heavier fragments (Steel 1984).

It is much easier to work with DNA on a solid support than in a gel and therefore the DNA is transferred onto either nitrocellulose paper or a nylon membrane by a method first described by Dr E M Southern (1975). This maintains the spatial relationship between

the DNA fragments generated by the gel electrophoresis. After "denaturing" the DNA with NaOH, to separate the two strands, the gel is placed on a glass plate which has been overlaid with a sheet of filter paper, with both ends dipping into a concentrated salt solution. The membrane is placed on top of the gel and pressed down onto it with a heavy weight. The salt moves down its concentration gradient carrying the DNA from the gel to the membrane, and the weight assists by gradually compressing the gel, forcing the DNA out. After this overnight "blotting" procedure, the membrane is exposed to ultraviolet light for 2 to 5 minutes. This links the single strand DNA to the membrane by covalent bonds.

The next step is to create a labelled DNA or RNA probe (i.e. millions of identical copies of a particular base sequence). The probes themselves are obtained by cloning the required piece of DNA as an insert in a virus-like vector which will grow in a bacterial host, usually E.coli.

The most common method of labelling a DNA probe is "nick translation"; a segment of double-stranded DNA is incubated with a mixture of 3 unlabelled nucleotides plus 1 nucleotide containing radioactive p^{32} atoms. The enzymes DNAase and DNA polymerase I are added. The DNAase introduces breaks ("nicks"), at random in one DNA strand and the DNA polymerase moves along that strand cutting out nucleotides and then replacing them, using the other strand as a template. In the course of this repair phase p^{32} labelled nucleotides are introduced into the DNA.

Another method used to label DNA probes to a higher specific activity with p^{32} is "random priming". The probe is first denatured by boiling, then a mixture of 3 unlabelled nucleotides, a p^{32} labelled nucleotide, bovine serum albumen, a primer, and the "Klenow"

fragment of DNA polymerase are added and left at room temperature for 3 to 5 hours. The "Klenow" fragment, using the single stranded probe as a template, moves along the probe, adding nucleotides onto the primer in the correct order and thus generates labelled probe sequences. This method is advantageous when only very small amounts of the probe are available.

The membrane bearing the imprint of the original DNA gel fragments, is immersed in a complex hybridisation solution, containing the labelled probe, shaken overnight at 68°C and then washed to remove unhybridised probe. If stringent washing conditions are used, the probe will hybridise only to these fragments of DNA on the filter, to which it is exactly complementary. After autoradiographic exposure (1 to 10 days at -70°C), one or more discrete bands appear on the film. These bands correspond to the DNA fragments on the membrane to which the probe has hybridised and the sizes of these fragments can be determined from the position of the bands.

At least 90% of the DNA in a human cell does not encode any protein product - i.e. does not consist of "genes". While this material is not necessarily devoid of function, it is evidently much more tolerant of variation in base sequences than the genes themselves. Hence, within the species there is much more polymorphism of the non-translated DNA than of the genes. One aspect of this polymorphism is the gain or loss of restriction enzyme cleavage sites, the positions of which can vary considerably from one individual to another. Thus when DNA is cleaved with a restriction enzyme and the Southern blot probed with a particular labelled sequence, the size of fragment bearing the complementary sequence is

quite likely to show some variation within the population. This "restriction fragment-length polymorphism" (RFLP) provides an enormous pool of genetic markers, since the positions of restriction sites are still sufficiently stable for the DNA fragment lengths to behave as alleles obeying simple laws of Mendelian inheritance. "Anonymous" DNA probes, recognising sequences that are not necessarily parts of structural genes, now represent a major resource in human gene mapping and genetic analysis. They may be used simply to increase the pool of "random" polymorphisms for the conventional "shotgun" approach to linkage studies or they may serve to provide polymorphic markers for adjacent candidate genes. The latter application ironically, tends to blur the distinction just made between "linkage" and "association" since the candidate gene, by definition, will be causally related to the disorder (implying association). Nevertheless, when the allelic forms of that gene are identified on the basis of restriction fragment-length polymorphism rather than by the putative mutation directly responsible for the disease, then any observed correlation between a <u>particular allele</u> and disease susceptibility will apply only within an individual family - i.e. we are dealing with true "linkage". Of course, in such an event, the logical course would then be to analyse the implicated gene in sufficient detail to permit direct identification of the crucial DNA lesion which would, in turn, provide a basis for population (as distinct from family based) screening.

MATERIALS AND METHODS

Materials and suppliers

BDH Analar

Hydrochloric Acid

Sodium Chloride

Ethylene Diamine Tetra Acetate

Sodium Dodecyl Sulphate

Chloroform

Isoamyl alcohol

Water saturated with phenol

Ammonium acetate

Isopropyl alcohol

Proteinase K

Ethanol

Acetic acid

Sodium Hydroxide

Sucrose

Sodium Citrate

Glucose

Potassium Acetate

Caesium chloride

Butan-1-ol

Calcium Chloride

Trichloro-acetic acid

Sodium acetate

Liquid paraffin

Glycerol.

SIGMA

RNA-ase A

Bromophenol blue

Agarose

Ethidium bromide

Polyvinylpyrrolidene

Sodium heparin

Sodium pyrophosphate

Salmon sperm DNA

Ampicillin

Tetracycline.

PHARMACIA

Dextran sulphate

Ficoll

AMERSHAM

Nick translation kit (No 8160SB)

Random priming kit (No RPM 1600Y)

P^{32} TTP

"Hybond N" Nylon membranes

WHATMAN

17mm filter paper

3mm filter paper

KODAK

XPan 4147 film

X-AR Omat film.

BOEHRINGER MANNHEIM

Tris

PLASTICS AND OTHER HARDWARE

STERILE UNIVERSAL CONTAINERS

Sterilin

GILSON

Eppendorf tips

TRANSATLANTIC PLASTICS

Layflat plastic

COSTAR

Spinex tubes

SCOT BOWTOWELS

Green towels

RESTRICTION ENZYMES USED

Enzyme	Buffer	Reference
Bam Hl	B	Roberts RJ et al Nature 265 p82 (1977)
Taq I	B	Sato S et al PNAS USA 74 p542 (1977)
Hind III	B	Old R et al J Mol Biol 92 p331 (1975)
Eco RI	H	Hedgpeth J et al PNAS 69 3448 (1972)
PST I	H	Brown NL FABS lett 65 p284 (1976)
Bgl I	H	Duncan CH et al J Bacteriol 134 p338 (1978)
MSP I	L	Waalwijk C and Flavell RA Nucl Acids Res 5 p3231 (1978)
Hinf I	H	Roberts RJ Nucl Acids Res 11 p135 (1983)
Ava II	A	Murray K et al Biochem J 159 p317 (1976)
Sac I	A	Roberts RJ Nucl Acids Res 11 p135 (1983).

Buffer Components	A	B	L	M	H
m/mols/litre					
Tris acetate	33	-	-	-	-
Tris-HCl	-	10	10	10	50
Mg-acetate	10	-	-	-	-
Mg Cl_2	-	5	10	10	10
K-acetate	66	-	-	-	-
NaCl	-	100	-	50	100
Dithioerythritol (DTE)	-	-	1	1	1
Dithiothreitol (DTT)	0.5	-	-	-	-
2-Mercaptoethanol	-	1	-	-	-
pH at 37°C	7.9	8.0	7.5	7.5	7.5

Probe Specifications

The chromosomal localisation and restriction enzymes used in this study are tabulated in the experimental data section. All these probes were grown in the MRC unit.

Harvey-ras; pEj

The 6.4kb Bam HI fragment of human genomic DNA containing the transforming "oncgene" from a bladder carcinoma cell line.

Vector-pBR 322

Tet^s, amp^r

Wash 0.1 SSC (Shih 1982)

Beta-globin;Pst B

A 4.4kb Pst I fragment of human genomic DNA.

Vector-pBR 322

Tet^r, amp^s

Wash 0.5 SSC (Orkin 1982).

PTH;pEB 3

A 2.1kb Eco RI fragment of human genomic DNA.

Vector pBR 322

Tet^r, amp^r

Wash 0.1 SSC (Mayer 1983).

Calcitonin;phT-3

A 590bp Pst I fragment isolated from a cDNA library made from thyroid medullary carcinoma.

Vector pAT 153

Tet^r, amp^s

Wash 0.5 SSC (Craig 1982).

Catalase;cat 4-1

An 1100bp Pst I fragment from a cDNA liver library.

Vector pKT 218

Tet^r, amp^s

Wash 0.5 SSC (Boyd 1986).

FSH-B;pRS 1.2

A 1.1kb Eco RI-SAC I fragment insert.

Vector pBR 322

Tet^r, amp^r

Wash 0.1 SSC (Watkins 1985; Glaser 1986)

λp11F9 D11 S49

A 15kb Bam H1 insert from E67.1 DNA.

Vector EMBL 3

Repeat sequences must be removed by stripping with sonocated placental human DNA.

Wash 0.1 SSC (Porteous 1987).

Apo A1;pA1

A 630bp Pst 1 fragment of cDNA from human liver.

Vector pBR 322

Tet^r, amp^s

Wash 0.1 SSC (Glaser 1988).

Int 2;pSS6

A 900bp Sac I fragment of cDNA from human placenta.

Vector pSP 64

Amp^r, tet^s.

Wash 0.1 SSC (Casey 1956).

Pepsinogen;pH PEP

A 705bp Pst 1 fragment isolated from a cDNA library from human gastric mucosa.

Vector pBR 322

Tet^r, amp^s

Wash 0.1 SSC (Taggart 1985, 1987).

Erb A2; pHeA2

A 1.9kb Eco RI genomic fragment from a human fetal liver cDNA library.

Vector pBr 312

Amp^r, tet^r

Wash 0.1 SSC (Jansson 1983, Gosden 1986).

MHC Class II;pll-B-4

A 1,080bp Eco RI/Pst I fragment from a human cDNA library.

Vector pBR 322

Tet^r, amp^s

Wash 0.1 SSC (Gustafsson 1984).

λMS 8;D5 S43.

A 7.0kb Bam Hl fragment in L 47.1.

Vector L 47.1

Wash 0.1 SSC (Wong 1987)

YNZ 22

A 1.7kb Bam Hl fragment from the cosmid isolated by a zeta-globin oligonucleotide.

Vector pBR 322

Amp^r, tet^s

Wash 0.1 SSC (Nakamura 1987).

Buffers and Solutions

Lysis buffer - 0.1M Tris HCl, 20mM NaCl, 1mM EDTA, 0.2% SDS.

Chloroform: isoamyl alcohol 24:1.

7.5M amonnium acetate (578.1g in 1 litre).

Resuspension buffer - 10mM Tris HCl, 150mM NaCl, 10mM EDTA.

10T-0.5E - 10mM Tris HCl, pH 7.5, 0.5mM EDTA

20xSSC - 876.6g NaCl, 441.2g Na_3 citrate in 5 litres.

0.1M Mg Cl_2

Cryogenic storage solution - 50:50 L-broth/glycerol.

Tris/EDTA/glucose - 500ml, 50mM glucose, 10mM EDTA, 25mM Tris HCl pH 8.0.

High salt solution - 3M K Ac, 2M acetic acid.

Dissolve K Ac in about 250ml of water, pH - 4.8 with acetic acid and then make volume up to 500ml.

20xTAE gel buffer - 484g Tris base, 114.2ml acetic acid, 200ml 0.5M EDTA in 5 litres.

Running buffer - 30% sucrose, 0.4% BPB.

Denaturing solution - 5g NaOH (0.5M), 219.15g NaCl (1.5M) in 2.5 litres.

Neutralising solution - 292.9g NaCl (2M), 394g Tris base (1M) in 2.5 litres.

Add HCl to pH 5.5.

L-broth. - 10g tryptone, 5g yeast extract, 5g NaCl, 1ml 1N NaOH in 1 litre.

T-broth. - 6g tryptone, 12g yeast extract, 2ml glycerol in 500ml.

Before innoculation add 50mls PO_4 buffer.

PO_4 buffer - 23gm KH_2PO_4, 125gm K_2HPO_4 in 1 litre.

NaOH/SDS - 0.2M NaOH, 1% SDS.

Wash buffer - 0.1SSC - 50ml 10% NaPPi, 25ml 20% SDS, 25ml 20xSSC in 5 litres.

0.5SSC - 50ml 10% NaPPi, 25ml 20% SDS, 125ml 20xSSC in 5 litres.

Ficoll solution - 12.5% Ficoll, SG 1.077.

50xDenhart's solution, - 5g Ficoll, 5g polyvinyl pyrrolidene, 5mg BSA in 500ml.

Alternative prehybridisation solution - 300ml 20xSSC, 50ml 50x Denharts, 12.5ml 2% SDS in 1 litre.

Dextran prehybridisation solution - 150ml 20xSSC, 50ml 50xDenharts, 12.5ml 20% SDS, 50g Dextran sulphate in 500ml.

Dulbecco's modified eagle medium (Dulbecco 1959; Smith 1960)

METHODS

Separation of lymphocytes (Boyum 1968)

1 One ml of whole blood was sent for high resolution chromosome banding as previously reported (Yunis 1976).

2 Centrifuge remaining blood at 2.5K for 5 minutes.

3 Pipette plasma off and discard.

4 Make volume up to 20mls with DMEM and shake.

5 Layer 10ml of this sample onto 10ml Ficoll solution in a sterile universal.

6 Add 10ml of lysis buffer to the other 10ml of blood in a 50ml graduated plastic tube (Falcon).

7 Centrifuge Ficoll solution at 2.5K for 15 minutes.

8 Pipette off visible lymphocyte band and then wash with DMEM.

9 Wash the remainder of red cells and granulocytes in the Ficoll sample, with DMEM then add to 10ml of lysis buffer.

10 Leave blood overnight in lysis buffer at $4^{o}C$.

11 Lymphocytes EB virus-transformed by Dr C M Steel as previously described (Diehl 1964).

DNA extraction from whole blood, tumour tissue, placentae and lymphoblastoid cell lines (Gross-Bellard 1972)

1 Add 10ml whole blood to 10ml lysis buffer and leave overnight at 4°C.

2 Add 10ml water saturated phenol, shake vigorously and centrifuge at 3K for 10 minutes.

3 Pipette off top layer, add 10ml of 24:1 chloroform isoamyl alcohol, shake and centrifuge at 3K for 10 minutes.

4 Pipette top layer off, add 5ml 7.5M ammonium acetate, and 20ml isopropyl alcohol, shake and leave overnight at-40°C.

5 Spool out any clumped, floating DNA with a glass rod.

6 Centrifuge at 3K for 15 minutes, then pour supernatant off and resuspend precipitate in 10ml resuspension buffer. Add any spooled out DNA on rod to this resuspension buffer.

6a If extracting from tumour or placentae the sample which was stored at -70°C is finely minced with a single sided razor blade as soon as it has defrosted enough to cut. Add 10ml resuspension buffer. If extracting from lymphoblastoid cell lines, add sample to 10ml resuspension buffer directly.

7 Add 100ul 20% SDS to final concentration of 0.2%.

8 Add 50ul of 10mg/ml RNAse to final concentration of 50ugm/ml.

9 Incubate with occasional agitation at 37°C for half an hour.

10 Add 100ul of 10mg/ml proteinase K to final concentration of 100 ug/ml.

11 Incubate with occasional agitation at 37°C for 1 hour.

12 Add 10ml of ice cold water saturated phenol and shake.

13 Leave on ice for 20 minutes.

14 Centrifuge at 3K for 10 minutes.

15 Pipette off top layer, add 10ml 24:1 chloroform isoamyl alcohol, shake.

16 Centrifuge at 3K for 10 minutes.

17 Pipette off top layer, add 5ml 7.5M ammonium acetate, 20ml 100% ethanol and shake.

18 Leave overnight at -40°C.

19 Spool out clumped DNA on glass rod then centrifuge at 3K for 15 minutes, pour off and keep supernatant at -40°C overnight.

20 Resuspend precipitate or spooled out DNA in 500ul of 10T.5E buffer.

21 Recentrifuge supernatant at 3K for 15 minutes.

22 Pour off supernatant and resuspend any precipitate in same 10T.5E buffer used in stage 20 in order to keep DNA as concentrated as possible.

23 Repeat 19, 20 and 21 till no further precipitate is seen.

Store DNA in 10T.5E at 4°C.

24 After 4-7 days in 10T-5E buffer, measure concentration of DNA in sample.

25 Add 10ul sample to 740ul distilled water, mix well and pipette into glass spectrophotometer cell.

26 Measure absorbence at 260nm and 280nm in PYE SP 6/400 spectrophotometer.

27 If DNA is clean A260/A280 should be 1.8.

28 Calculate concentration. A reading of 1 at 260nm = a DNA concentration of 50mg/ml. Therefore reading of absorbance x 50 x 75 = concentration in mg/ml.

DNA digestion

1 Calculate the volume of 10ug of genomic DNA.

2 To 10ug of DNA add 4ul of the appropriate buffer (as listed in the Materials section) in a stoppered Eppendorf tube (Treff).

3 Add sterile distilled water to final total volume of 38ul.

4 Add 2ul (20units) of restriction enzyme.

5 Mix thoroughly with Whirlimix (Jencons).

6 Spin briefly in an Eppendorf centrifuge (Sorvall microfuge).

7 Incubate overnight at 37°C.

- Note if digesting with Taq I, incubate at 65°C.

8 The following morning spin briefly in Eppendorf centrifuge to remove condensed water from lid.

9 Add further 2ul of restriction enzyme.

10 Mix throughly with Whirlimix.

11 Spin briefly in an Eppendorf centrifuge.

12 Incubate for further 3-5 hours at 37°C to ensure complete digestion (or 65°C if Taq I used).

13 Spin briefly in Eppendorf centrifuge.

14 Add 10ul of running buffer.

15 Mix with Whirlimix.

16 Spin briefly with Eppendorf centrifuge.

17 Samples are now ready to load on agarose gel.

Electrophoresis in agarose gel (McDonell 1977, Southern 1979)

1 All our samples are run in full size (20cmx25cm) 500ml gels.

2 Make gel by dissolving 4g purified agarose in 500ml 1xTAE buffer, weigh flask, stirrer, agarose and buffer.

3 Boil gel.

4 Reweigh and add distilled water to original weight.

5 Cool gel, while stirring, to 60°C.

- If using high frequency cutting restriction enzymes (Taq I, MSP I) use 1% agarose gel; i.e. 5gm agarose in 500ml TAE buffer.

6 Prepare electrophoresis tank (BRL Model H4) by ensuring tray is flat, stick autoclave tape to both ends of tray, and put correct comb (i.e. with appropriate number of slots) in place.

7 When gel has cooled to 60°C, pour gel into tray removing any trapped air bubbles with a pipette.

8 Leave gel until it has completely set.

9 Remove autoclave tape from end of tray.

10 Pour in a sufficient volume of 1xTAE buffer to completely submerge gel.

11 Remove comb carefully.

12 Slide a piece of coloured paper below wells to improve visibility.

13 Load one sample into each well.

14 If a molecular weight marker is needed, load 0.4mg of Hind III digested λDNA into the appropriate well.

15 Connect up pump (Crouzet 10rpm) to circulate buffer through a beaker of ice. This is important to get lanes as straight as possible by preventing buffer heating up or accumulation of ions in various compartments of the tank.

16 Connect up tank, remembering DNA runs to anode.

17 Run at 60V for 16-48 hours.

- resolution of Bam HI fragments for probing with Harvey Ras requires run of 48 hours. For the other probes used, gel ran for 16-20 hours.

18 Switch off current, mark one corner of gel for later orientation of gel and membrane and immerse gel in 1 litre TAE buffer, with 50ul of 10mg/ml ethidium bromide to final concentration of 0.5ug/ml for 30 minutes with occasional agitation (Sharp 1973).

19 Pour off buffer and destain with 1 litre distilled water for 10 minutes.

20 Place gel on UV transilluminator (UVP (Inc)) and wearing a plastic visor look at gel, to detect incomplete digestion or squint channels.

21 Photograph gel using bellows camera, orange filter and Kodak X Pan 4147 film.

22 Denature DNA in gel by immersion in 1.25 litres of denaturing solution with gentle agitation for 45 minutes.

23 Remove gel and neutralise by immersion in 1.25 litres of neutralisation solution with gentle agitation for 1 hour.

- gel is now ready for blotting procedure.

Southern blotting (Southern 1975)

1 Prepare blotting apparatus by placing a piece of glass (0.5cm x 23cm x 48cm) on a plastic tray (30 x 45cms) containing 1-2 litres of 20 x SSC buffer. On glass plate place a piece of 17mm filter paper, with both ends immersed in 20 x SSC buffer.

2 Thoroughly wet filter paper with 20 x SSC buffer and remove all trapped air bubbles by rolling glass pipette over paper.

- when not in use tank should be completely covered with cling film to reduce evaporation.

3 Place a piece of 3mm filter paper on the 17mm paper, thoroughly wet paper with 20 x SSC buffer and remove trapped air bubbles.

4 Remove gel from neutralising solution, shake off excess solution, place on filter paper and remove trapped air bubbles.

5 Place cling film around gel, to completely cover tank, reducing evaporation and preventing crystallisation of the very concentrated salt solution.

6 Place a piece of Hybond N (Amersham) (20 x 23cm) over gel, and remove trapped air bubbles.

7 Place two pieces of dry 3mm filter paper over Hybond N, then a pile of white paper towels (Kleenex) then arrange green paper towels (Scott Bowtowels) on this pile to give complete coverage of pile.

- this insures equal transfer of the DNA onto Hybond N.

8 Place plastic tray on towels, then two bricks and a 1kg weight.

9 Leave overnight.

10 Remove all towels and the two pieces of 3mm filter paper.

11 With ballpoint pen, mark heights of slots and cut corner on Hybond, and name and date of filter.

12 Peel Hybond membrane off gel and discard gel and single piece of 3mm filter paper, and recover whole blotting apparatus with cling film.

13 Place Hybond membrane on a clean UV transilluminator (IVP (Inc)), DNA side down and illuminate for 4 minutes.

- this covalently binds the DNA to the membrane.

The transilluminator must be calibrated for optimum length of illumination as over exposure can break the covalent bonds already formed, making it impossible to reprobe the membrane effectively.

14 The membrane is now ready for the first step of the hybridisation procedure.

GROWING PROBES

Preparation and transformation of competent cells (Mandel 1970; Dagert 1974)

Preparation

1 Inoculate a single colony of E.coli DH1 into 100mls of sterile L-broth, without antibiotics.

2 Shake for 5 hours at 37°C.

3 Measure optical density at 260nm in Pye SP6/400 spectrophotometer.

4 When OD reaches 0.5, centrifuge at 3K for 10 minutes at 4°C in Sorval RC 5B).

5 Discard supernatant and resuspend cells in 25ml precooled 0.1M $CaCl_2$.

6 Centrifuge at 3K for 10 minutes at 4°C.

7 Discard supernatant and resuspend pellet in precooled 0.1M $CaCl_2$.

8 Centrifuge at 3K for 10 minutes at 4°C.

9 Discard supernatant and resuspend pellet in 5ml 0.1M $CaCl_2$.

10 To store, dispense into Eppendorf tubes (Treff) and freeze immediately in a flask of liquid N_2.

- for cryogenic storage at -70°C, resuspend pellet in 500ul of 50:50 L-broth/glycerol.

Transformation

1 Add 0.1ug plasmid DNA to 100 1 of competent DH1 E.coli in an Eppendorf tube and mix gently on ice.

2 Leave on ice for 30 minutes.

3 Place in 42°C water bath for 3 minutes to heat-shock cells.

4 Add 100ul L-broth without antibiotics and incubate at 37°C for 1 hour.

5 Plate 100ul onto an agar plate containing the appropriate antibiotic, and spread with a glass spreader.

6 Incubate at 37°C upside down overnight.

Probe growing and extraction (Silhavy 1984)

1 Plate out a colony picked from a stab or from cryogenic storage onto an agar plate with appropriate antibiotic at concentration of 50 ug/ml.

2 Incubate overnight at 37°C.

3 Pick one single colony and infect 25ml of T-broth containing antibiotic at 50ug/ml in a 50ml blue tube (Falcon). Make 3 similar tubes.

4 Incubate the 4 tubes overnight at 37°C in orbital shaker (Gallenkamp) shaking at 210rpm.

5 Centrifuge at 3.5K for 15 minutes to precipitate bacteria.

6 Discard supernatant.

7 Resuspend pellets from 2 tubes in 2mls Tris/EDTA/glucose and transfer into 50ml tube (Sarsted).

8 Add 4mls NaOH/SDS and mix well.

9 Leave for 10 minutes on ice.

10 Add 3ml high salt solution, and mix well.

11 Leave for 10 minutes on ice.

12 Centrifuge at 10K for 20 minutes at 4°C in Sorval ultracentrifuge (RC5 B).

13 Pour precipitate through muslin into clean 50ml tube (Sarsted).

14 Add 0.6 volumes (5.4ml) of isopropyl alcohol and mix well.

15 Leave for 30 minutes at -40°C.

16 Centrifuge at 10K for 20 minutes in Sorval ultracentrifuge.

17 Discard supernatant.

18 Resuspend precipitate from both tubes in 3mls 10T.5E buffer and transfer to 5ml plastic tubes (Sarsted).

19 Add 20ul of 10mg/ml RNAse to final concentration of 100mg/ml.

20 Incubate at 37°C for 30 minutes.

21 Add 2ml water-saturated phenol and mix well.

22 Centrifuge at 3K for 10 minutes.

23 Pipette off top layer, add 2ml 50:50 phenol chloroform and mix well.

24 Centrifuge at 3K for 10 minutes.

25 Pipette off layer. Add 2ml 24:1 chloroform isoamyl alcohol and mix well.

26 Centrifuge at 3K for 10 minutes.

27 Pipette off top layer, add 1ml 7.5M ammonium acetate and 4mls absolute alcohol. Mix well.

28 Leave overnight at -40°C.

29 Centrifuge at 10K for 20 minutes.

30 Resuspend precipitate in 100 1 of 10T.5E. Store at -20°C.

31 Run 2ul of this solution on a minigel, (BRL H6), 0.8% agarose with 0.5 gm/ml of ethidium to see how clean probe is.

- if the probe is clean enough, label as in Section 8.

Cleaning up probe

There are two widely used methods

a) Gel electrophoresis

b) Caesium chloride gradients

We prefer to use gel electrophoresis.

a) Gel electrophoresis

1 Make 150ml 0.8% agarose gel without ethidium-bromide, in a midi tank (BRL H5).

2 Pour gel, using 12 well comb, not a 2 well, preparative comb.

3 Pour in TAE buffer to cover gel and remove comb carefully.

4 Add 20ul of running buffer to 100ul of probe solution. Whirlimix and spin briefly in Eppendorf centrifuge.

5 Load gel, filling each slot as fully as possible.

6 Load 0.4mg of Hind III λDNA in one slot, to act as molecular weight marker.

7 Run gel at 60V for 3-5 hours, depending on molecular weight of plasmid and insert being cleaned up. It is essential to run the probe far enough so that a definite band can be seen, but running too far results in broadening of the band, and the final concentration of the probe will be very low.

8 Stain gel as in Section 4, steps 18-20.

9 Excise appropriate band with clean single-edged razor blade, removing all excess gel.

10 Mince gel slice with razor and scoop gel into top of spinex tube (Costar).

11 Centrifuge spinex tube in Eppendorf centrifuge for 30 minutes.

12 Recover liquid at bottom of spinex tube and check how clean and how concentrated probe is by running another mini gel (BRL H6).

This method can also be used to isolate an insert, which has been cut out of a plasmid.

Caesium chloride gradient (Yamamoto 1970)

1 After step 29, resuspend precipitate in 19.26ml 10T.5E buffer.

2 Weigh out 21.30g of Caesium chloride into a 30ml polyallomer tube (Sorval).

3 Add 2.04ml of 10mg/ml ethidium bromide and mix well.

4 Pipette the resuspended probe in a layer onto the top of the tube, and fill tube with a layer of liquid paraffin.

5 Tubes have to be accurately balanced to 0.1gm.

6 Centrifuge at 40K for 18 hours at 20°C in Sorval OTD 65 ultracentrifuge.

7 Using the ultraviolet transilluminator (IVP Inc) a bright band halfway down the tube can be seen.

8 Pipette off band in to a 10ml plastic tube (Sarsted).

9 Add equal volume (5-10ml) water-saturated butan-1-ol and mix well.

10 Leave to stand in ice for 5 minutes.

11 Pipette off top layer and discard it.

12 Repeat steps 9-11 until the top layer is completely clear.

- this procedure removes the ethidium-bromide from the probe.

13 Add 2.5 volumes (25ml) of 75% ethanol.

14 Leave at -70°C for 30 minutes.

15 Thaw solution, then centrifuge at 8K for 20 minutes in Sorval ultracentrifuge (RS 5B).

16 Discard supernatant.

17 Resuspend precipitate in 100ul 10T.5E.

18 Run 2ul on mini gel (BRL H6) as in step 12 above.

Probe Labelling (Rigby 1977)

a) "Nick" translation

The majority of probes which have been used in this work were labelled by nick translation using the Amersham kit (No 8160SB).

1 Add 0.1ug of probe DNA, 5ul of unlabelled nucleotide solution (Solution A4), 2ul of P^{32} TTP (20 Ci) 5ul of DNAse/DNA polymerase (Solution C) and sterile distilled water to give a final volume of 50ul

2 Mix gently.

3 Incubate at 16°C in water bath in cold room for 3-5 hours.

4 To estimate % incorporation; pipette 1ul of solution onto a Whatmann B microfibre filter paper, place in a scintillation vial (Motil platics), count for 0.1 minutes in Tricarb scintillation counter (Packard model 3330) (Struhl 1987).

5 Remove filter paper, place on Buchner flask with vacuum pump, and wash with 30ml of 5% TCA.

6 Place filter in scintillation vial containing 10ml 5% TCA and count for 0.1 minute.

$$\% \text{ incorporation} = \frac{\text{count in 4}}{\text{count in 6}} \text{ and should be 30-50\%-}$$

Specific activity = count in 6 x 10 (as counted for 0.1 minute) x 4 (window on counter) x 10 (as 0.1ug as probe DNA was used) x 50 (reaction volume) and should be around 5×10^8 cpm/ug.

If dextran prehybridisation solution is used the labelled probe must be separated from unincorporated nucleotides by ethanol precipitation.

7 Add 20ul of 10mg/ml sonicated salmon sperm DNA solution to act as a carrier for the probe.

8 Add 1/50 volume (1-2ul) of 3M NaOAc.

9 Add 2 volumes (140ul) of 100% ethanol.

10 Mix briefly and leave at -40°C for 30 minutes.

11 Spin in Eppendorf centrifuge for 30 minutes.

12 Pipette off supernatant and discard, taking care not to disturb precipitate.

13 Resuspend precipitate in 500ul of 10mg/ml sonicated salmon sperm DNA.

14 Place in boiling water bath for 10 minutes to denature probe.

- probe is then ready to be added to membrane.

If the alternative prehybridisation solution is used, there is no need to remove unincorporated nucleotides.

15 Add 500ul of 10mg/ml sonicated salmon sperm DNA, boil for 10 minutes and the probe is then ready to be added to the membrane.

Random priming (Jacobsen 1974; Feinberg 1983)

If the insert has been cut out of the plasmid, or very small amounts of probe are available, random priming is the labelling method of choice. We have routinely used the Amersham random priming kit (RPN 1600Y).

1 If circular plasmid is used, it must be linearised by digestion with an appropriate restriction enzyme, i.e. one will not cut out the insert, but will cut at one site in the plasmid.

2 Add 25-200ng of probe, 5ul of unlabelled nucleotide solution, 5ul BSA/primer solution, 5ul of buffer, 2ul of P^{32} TTP (20 Ci). 5ul of "Klenow" fragment of DNA polymerase, and sterile distilled water to give final reaction volume of 50ul.

3 Leave for 3-5 hours at room temperature, then test percentage incorporation as before in steps 4 onwards.

- remove unincorporated nucleotides by ethanol precipitation as above.

Hybridisation (Southern 1975, Thomas 1980)

1 Make bag for membrane by heat-sealing a length of double-sided plastic (Transatlantic Plastics).

2 Cut off corner of membrane previously marked with ballpoint pen as the pen marks will be removed during this process. Place membrane in bag and seal 3 sides with 2 seals per side.

3 Add 40ml of prehybridisation solution, preheated to 65°C in water bath.

- we have used two prehybridisation solutions, as listed in the materials section.

Dextran prehybridisation solution should not be used with the Harvey-ras probe.

4 Remove as many air bubbles as possible, and heat-seal the top of the bag twice.

5 Immerse membrane in shaking water bath at 65°C for at least 30 minutes.

6 Remove plastic bag from water bath and cut a small incision through one layer of the plastic at the top corner and insert a sterile blue Eppendorf tip (Gilson) into the hole- to ensure that both layers of plastic have not been cut.

7 Remove blue tip and pipette in all the probe solution.

8 Remove air bubbles and reseal bag.

9 Wash outside of bag with water.

10 Ensure that probe has gone into the bag by placing the bag in front of a Geiger counter (Mini Instruments Ltd).

11 Return bag to 65°C water bath and leave overnight.

12 Pour 500ml of wash buffer at 65°C into plastic tray.

- we have used 0.1 SSC wash buffer for the majority of probes, and 0.5 SSC buffer for a few.

13 Remove plastic bag from water bath and cut off three sides under level of wash buffer.

14 Slide out membrane and discard plastic bag.

15 Pour away wash buffer and add a further 500ml of wash buffer.

16 Agitate tray in shaking oven (Gallenkamp) at 65°C for 10 minutes.

17 Pour away wash buffer and add a further 500ml and replace in shaking oven for 10 minutes.

18 Repeat step 17 again.

19 Remove membrane from wash buffer and place between two sheets of clean, dry 3mm Whatmann's filter paper for 30 seconds.

20 Place membrane on a clean sheet of 3mm filter paper, DNA side up, cut to the size of the membrane, remembering to cut off the corner of the paper to allow later orientation.

21 Wrap membrane and filter in cling film, making three cuts in the right-hand side of the cling film and squeezing out any excess air.

22 Place wrapped membrane in X-ray cassette.

23 Preflash a piece of X-AR Kodak Omat film, with one thumb over lower corner, to allow orientation of autoradiograph.

24 Place film exactly over membrane, (in total darkness), and close X-ray cassette firmly.

25 Leave overnight at -70°C.

26 Remove film (in total darkness) and develop it in autoradiographic developer (RGII Fugi X-ray film processor).

27 The bands are likely to be very faint, but it will be clear if the membrane has been properly washed.

28 Repeat steps 23 and 24.

29 Leave at -70°C for 10-14 days, then develop second film as in step 26 to give final autoradiograph.

Reprobing a Hybond-N membrane

1 Remove cling film from membrane.

2 Place membrane in 400ml of boiling 0.1% SDS.

3 Switch off hot plate and allow solution to cool.

4 Repeat steps 19-25 to ensure that the first probe has been washed off completely.

- if the filter is clean it can be reprobed with a different probe following the method already described.

Clinical Material

Tumour samples were collected from 100 patients undergoing Patey mastectomy or wide local excision, immediately frozen on dry ice, and stored at -70°C until DNA extraction.

All patients had presented with palpable breast lumps and were referred by their general practitioners to the breast clinic in the Royal Infirmary of Edinburgh. Patients with T4 tumours or with distant metastases at presentation were excluded, as they were usually treated by chemotherapy in the first instance. All patients underwent either axillary lymph node sampling or axillary clearance. 20ml of venous blood was collected from these patients (with their informed consent) and transferred to a sterile Universal with 200U of sodium heparin. Similar 20ml samples were collected from both affected and unaffected members of the large kindred described in the section on Experimental Data. Blood samples were immediately separated and lymphoblastoid cell lines set up as in the Methods section.

One portion of tumour was placed on ice and the oestrogen receptor protein concentration immediately assayed by Dr R A Hawkins (Lister Laboratories of the University Department of Clinical Surgery), by a saturation analytical method with separation of free and bound hormone using Dextran-coated charcoal adsorption as previously described (Hawkins et al 1981).

Samples from patients who had received a synthetic anti-oestrogen Tamoxifen were also examined by enzyme immunoassay (ER-EIA Kit, Abbott laboratories, as in Leclerq 1984). Tamoxifen interferes with the former but not the latter assay.

The greatest diameter of these excised tumours was accurately measured in millimetres by Drs T J Anderson and J J Going of the Department of Pathology. They were classified into histological types (Page 1988), examined for vascular invasion, lymphatic invasion and in situ carcinoma.

Lymph nodes were also examined histologically for metastases.

Information on age at presentation, menopausal status, family history, clinical tumour staging, and drug history was available on all these patients.

Samples from 100 placentae were collected with the cooperation of the Obstetrics and Gynaecology Department of the Western General Hospital and stored at $-70^{o}C$ until DNA extraction as in the Methods section.

Details of the pedigree of the large kindred described in the Experimental Data section were ascertained, drawn and verified by Mrs S Collyer and Mrs R De Mey in the Registry of the MRC CAPCU. The MCF-7 cell line used was kindly donated by Dr W R Miller (Lister Laboratories).

EXPERIMENTAL DATA

1 Bam H1 Harvey-ras alleles.

The initial aim of the study was to verify or refute the claims of Krontiris and colleagues (Krontiris 1986) that patients with cancer (specifically breast cancer) show an increased incidence of rare Harvey-ras alleles in their constitutional (i.e. blood) DNA. At the same time DNA was extracted from a series of breast tumours to see if there were detectable somatic changes at the Harvey ras locus.

The four common Bam H1 Harvey ras alleles are seen in Fig. 5.

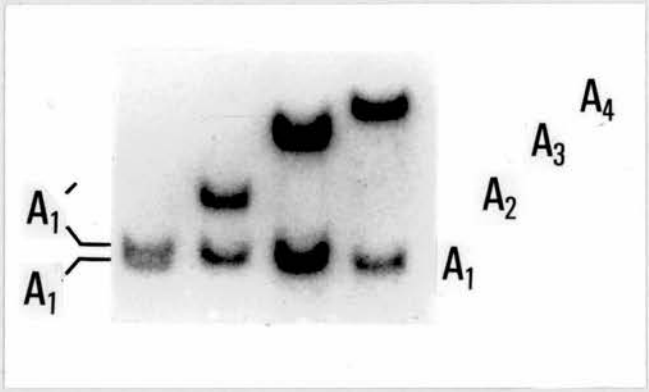


Fig 5 Alleles of c-Ha-ras (Bam H1 digests) from four placental DNA samples. The left hand track of this Southern blot contains a 'doublet' of allele A, and the rare variant A_1'.

In common with other authors the restriction fragments are arbitrarily indexed in ascending order of molecular weight. A1 is 6.9kb, A2 is 7.6kb, A3 is 8.0kb and A4 is 8.3kb. A3 and A4 are fairly close together and as is clear from Figure 1, the difference in molecular weight between the common A1 allele and its rare variant A1' is small. We have routinely run these gels for 48 hours with the buffer being pumped through ice to give clear, repeatable resolution of these fragments. We have also extracted DNA from a breast cancer cell line MCF 7, which is homozygous for a rare A1' allele, as seen in Figure 6.

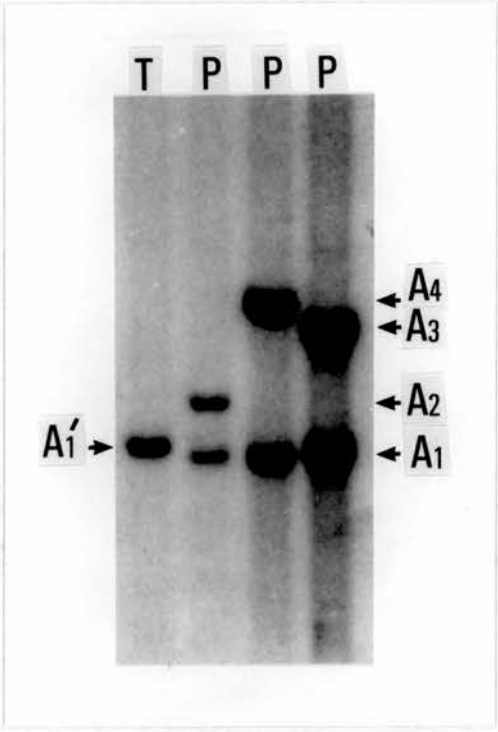


Fig 6 Bam H1 digests of DNA extracted from MCF 7 cells (T) with three placental controls (P) probed with c-Ha-ras.

We run standard placental samples containing 4 common alleles on both sides of each gel, and so we are confident that our system will reproducibly detect rare alleles. Table 1 shows the relative frequencies of these alleles in 100 breast cancer patients and 100 placentae.

Table 1

The distribution of Bam H1 alleles at the Harvey ras locus in 100 breast cancer patients and 100 placentae

	100 Breast cancer patients		100 Placentae	
	Number	%	Number	%
A_1	126	63.0	135	67.5
A_2	25	12.5	27	13.5
A_3	23	11.5	19	9.5
A_4	19	9.5	15	7.5
Rare Alleles*	7	3.5	4	2.0

In agreement with other authors, rare alleles can be identified in the normal population, but there is no significant difference between breast cancer patients and controls in the frequencies of these rare alleles; nor is there any shift in the distribution of common alleles between the two groups.

Harvey ras allelic loss in tumour DNA

While completing the analysis on Section 1, we noted that in several of the heterozygous tumours, 1 allelic band was much darker than the other as illustrated in Figure 7.

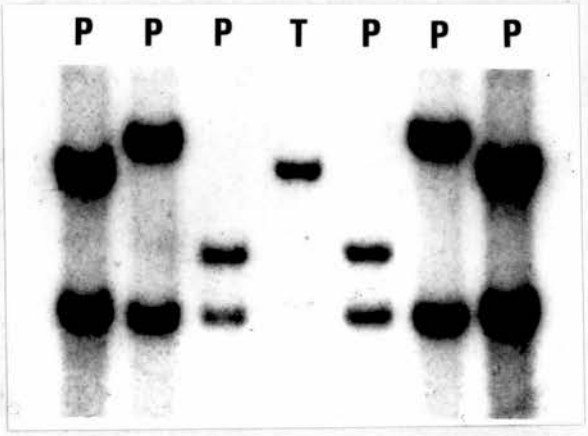
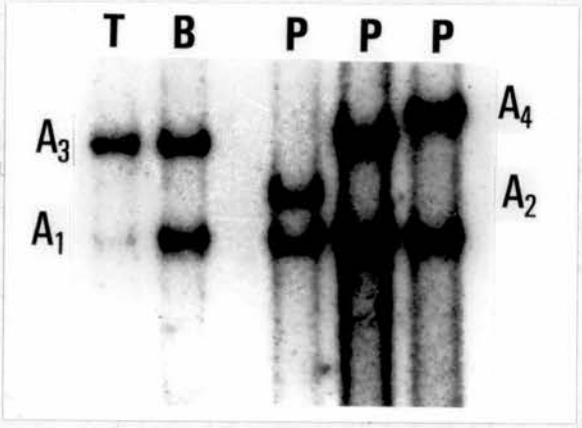


Fig 7 Bam H1 digests of DNA extracted from a breast tumour (T) with placental controls (P) as before probed with c-Ha-ras. Note that the lower tumour allele band (A_1) is much less dense than the upper (A_3).

The relative intensities of the bands in different gel tracks suggested that there had been no gross amplification of a Harvey ras gene in any of the tumours. An alternative explanation for the evident imbalance between the two alleles in some DNA preparations is that the tumour cells have lost one allele and the fainter band seen results from contamination of the tumour sample by non-neoplatic cells. This was supported by comparing paired tumour DNA and white blood cell DNA samples from the same patient, as seen in Fig. 8.

Fig 8 Bam Hl digests of tumour (T) and blood leukocyte (B) DNA from the same patient, probed with c-Ha-ras and compared with three placental controls (P). Note that alleles A_1 and A_3 are of equal intensity in B, but allele A_1 is almost absent from the tumour sample.



However, if there was minimal infiltration by non-neoplastic cells, the tumour sample might appear homozygous i.e. one allelic band was lost so completely that it was not visible. In our study, 61 tumours were heterozygous of which 10 had clear loss of one allele. 39 tumours were "apparently" homozygous, but when we examined the white blood cell DNA in these individuals 4 were constitutionally heterozygous. The total collection of tumours could be subdivided as follows.

1 No allelic loss at the Harvey-ras locus - 51 tumours.

2 Loss of one allele - 14 tumours.

3 Uninformative, because the patient was constitutionally homozygous - 35 tumours.

Thus, 14 out of 65 informative tumours have lost one Harvey-ras allele.

Clinico-pathological associations of Harvey ras allelic loss

There was no preferential loss or retention of any of the four common alleles, and our present analysis does not allow us to determine the maternal or paternal derivation of a lost allele.

In an analysis of the 65 tumours informative at the Harvey-ras locus, we found no significant correlation between allelic loss and menopausal status, age at presentation or history of an affected first degree relative.

However, as shown in Table 2 there was a significant correlation between loss of a Harvey-ras allele and paucity of oestrogen receptor protein, absence of oestrogen receptor being a well-recognised index of poor prognosis (Croton 1981; Moore 1983; Williams 1987; Shek 1987).

TABLE 2 - RELATIONSHIP BETWEEN LOSS OF A HARVEY RAS ALLELE AND OESTROGEN RECEPTOR LEVEL IN 61 BREAST TUMOURS

	ER poor/-ve ⩽20fmol/mg protein	ER moderate/rich > 20fmol/mg protein (20)
Allelic Loss	8	6
No Allelic Loss	10	37

p= 0.02 by Fishers exact test

4 samples from patients on Tamoxifen produced a doubtful assay and were therefore excluded.

There was also a significant correlation between tumour size and allelic loss as shown in Figure 9.

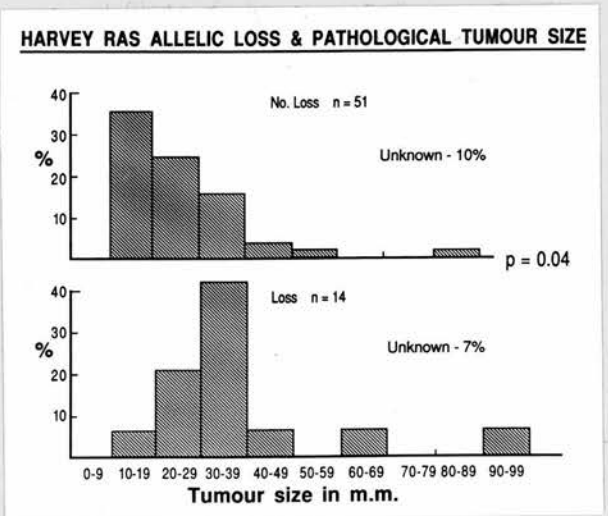


Fig 9 Distribution of primary tumour size (greatest diameter in mm) in relation to Ha-ras allele status.

TABLE 2a

	Ras loss	No Ras loss	Unknown	X^2
+ve lymph node involvement	2/11	18/45	9	3.2
+vascular invasion	2/14	12/51	0	0.84
named histological type	2/14	12/51	0	0.84

All values are insignificant by the X^2 test, with Yates correction, if appropriate.

There was no significant correlation between allelic loss and pathological lymph node involvement, vascular invasion, lymphatic invasion, or histological type of tumour.

Mechanism and extent of loss of a Harvey ras allele

Several mechanisms could be responsible for the finding that 21% of primary breast carcinomas have lost one Harvey-ras allele. These include a deletion, mitotic recombination, or loss of a whole chromosome as explained in the Introduction (p7). In an attempt to clarify which mechanism is most likely to have occurred in our material, we have examined several other loci on chromosome 11. The numbers involved in this part of the study are smaller, as we had to use DNA from patients' lymphoblastoid cell lines for the multiple digestions required.

We have examined 3 loci on 11q as detailed in Table 3.

TABLE 3

Gene or probe	Designation	Localisation	Restriction Enz	Ref	No Examined	No Informative	No Lost
APOA 1	pBA1	11q23	Sac I	(1)	20	0	-
Int2	pSS6	11q13	Bam H1	(2)	20	0	-
Pepsinogen	pH PEP	11q12	Eco RI	(3,4)	24	20	0

(1) Glaser 1988
(2) Casey 1986
(3) Taggart 1985
(4) Taggart 1987

Two loci were completely uninformative in our patients, but the third, Pepsinogen, provided good evidence that a whole copy of chromosome 11 has not been lost in these tumours, and we therefore turned to a more detailed analysis of 11p.

We have examined 5 loci on 11p outside Harvey-ras, as detailed in Table 4.

TABLE 4

Gene or probe	Designation	Localisation	Restriction Enz	Ref	No Examined	No Informative	No Lost
β-globin	Pst-β	11p15	Bam H1	*1	20	12	4
PTH	pEB 3	11p 13-15	Pst 1	*2	19	9	5
Calcitonin	phT-B3	11p 15-4	Taq 1	*3	20	4	2
FSH-β	pRS 1-2	11p 13	Hind III	*4,5	22	8	3
D11S49	λp11 F9	11p 13	Bgl 1	*6,7	19	16	5
				Total		49	19

*1 - Deisseroth et al 1978
*2 - Naylor et al 1982
*3 - Hoppener et al 1984
*4,5 - Glaser et al 1985; Watkins et al 1985
*6,7 - Porteous et al 1987; Boyd et al 1989

Representative autoradiographs from some of these probes are illustrated in Fig 10.

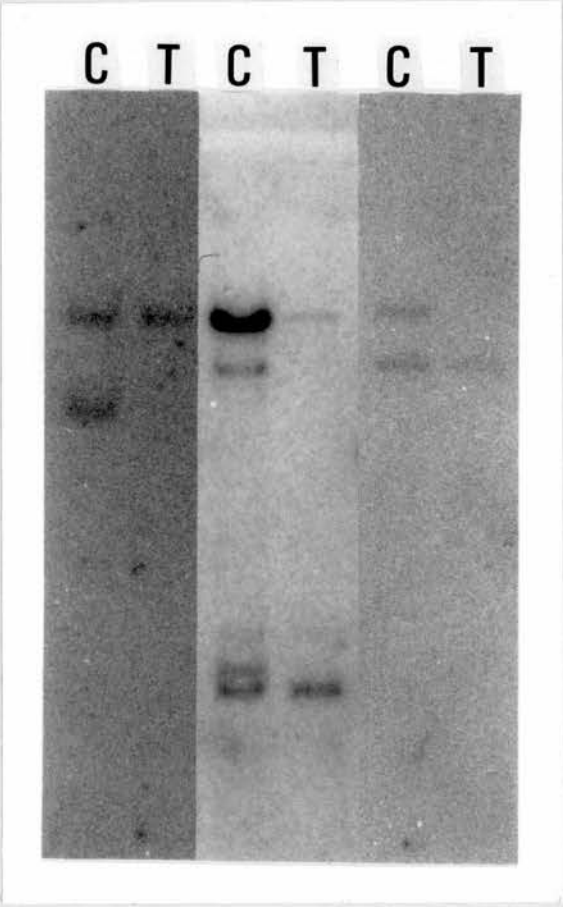


Fig 10 Paired digestions of DNA from tumour (T) and lymphoblastoid cell lines (C) from 3 patients, probed with (i) PTH (pST1) (ii) β-globin (Bam H1) (iii) Calcitonin (Taq). Note loss of one allele in each tumour.

Heterozygosity was found on a total of 49 occasions and the corresponding tumours had lost an allele in 19 cases (38.8%). 19 tumour/cell line pairs have been fully characterised for all five loci, as tabulated in detail below.

TABLE 5

DETAILS OF ALLELIC LOSSES ON 11p IN 19 TUMOUR/CELL LINE PAIRS

Tumour/ cell line pair	Ha-Ras	β-globin	PTH	Calcit	FSH-β	λP11F9
1	a-	u	a-	a-	a-	a-
2	a-	a-	u	u	a-	u
3	a-	u	u	a-	u	u
4	a-	u	u	ab	ab	u
5	a-	u	u	u	u	ab
6	a-	u	u	u	u	ab
7	a-	ab	u	u	u	ab
8	a-	ab	a-	u	a-	ab
9	ab	u	u	u	u	a-
10	ab	u	u	u	u	ab
11	ab	ab	ab	u	u	ab
12	ab	ab	ab	u	u	ab
13	u	ab	u	u	ab	a-
14	u	ab	a-	u	u	a-
15	u	u	a-	u	u	a-
16	u	ab	u	u	u	ab
17	u	u	ab	u	ab	u
18	u	a-	u	u	u	ab
19	u	a-	u	u	u	ab

a = informative and lost
ab = informative, not lost
u = uninformative (constitutionally homozygous).

Of the 19 pairs, 12 were informative for Harvey-ras, of which 8 showed allele loss (1-8). Three of the 8 (1,2,3) showed loss at all informative loci on 11p consistent with loss by mitotic recombination below 11p 13.

Four of the 8 (4,5,6,7) were uninformative or showed no loss at any of the other 11p loci, while the remaining tumour (8) showed allele loss at Harvey-ras, PTH and FSH B, but not D11 549 and more significantly beta-globin. This suggests that a double event (involving inversion or deletion and mitotic recombination) has taken place in this tumour as the chromosomal localisation of Harvey-ras PTH and beta-globin relative to each other is well-established by physical and genetic linkage analysis (Naylor 1982; Desseroth 1978).

In one of the 4 tumours (9) which were informative for ras, but showed no loss, there was loss of the 11p13 marker D11 S49, and similarly amongst the 7 tumours uninformative for ras (13-19) we found allelic loss at D11 S49 in one (13), where FSH B and beta-globin were both informative but showed no loss. Two further tumours showed loss at D11 S49 and also at PTH with intervening loci being uninformative (14,15).

This detailed analysis demonstrates that no specific locus or region was ubiquitously lost.

Of these 19 tumour/cell line pairs, 10 (53%) had lost an allele at one or more locus, (excluding Harvey-ras) on 11p. This figure must be treated with caution, as 8 out of the 19 pairs were known to have lost Harvey-ras, and the data in Section 2 suggests Harvey-ras loss in 21% of primary tumours. However, it is clear that there is a substantial frequency of DNA lesions on 11p in primary breast tumours.

Specificity of allelic loss on 11p

In order to assess whether allelic loss is a generalised phenomenon throughout the genome, we have examined several other loci using probes that are highly polymorphic, and therefore likely to be informative in a high percentage of individuals, as detailed in Table 6 with representative autoradiographs below.

TABLE 6

Gene or probe	Designation	Localisation	Restriction Enz	Ref*	No Examined	No Informative	No Lost
Pepsinogen	pH PEP	11q12	EcoR I	1,2	24	20	0
Erb A	pHcA2	17q21.3	Bam H1	3	20	11	0
MHC Class II	p11-B-4	6q21.3	EcoR 1	4	21	21	0
λMS8	D8 S43	5q34qter	Hinf 1	5,6	18	15	1
				Total		67	1

Adding in the data on 11q from the previous section we find loss in only one of 67 informative loci, so it is not a generalised phenomenon. As a continuation of this work, we also used the minisatellite locus-specific probe, YNZ 22, mapping to 17p, with the restriction enzyme MSP I (as detailed in the Methods section).

The resulting autoradiographs were very complex, with a large number of bands, although it did appear that several tumours had lost one or more bands. Whether this was due to incomplete digestion was unclear. Examining a panel of normal placental DNAs, confirmed that, although it was definitely possible to identify different genotypes, it was very difficult to identify allelic bands as seen in Fig 12. A much more readily analyzable pattern was produced by using a different restriction enzyme, Taq I and it became clear that loss of the YNZ 22 locus on 17p was common in breast tumours.

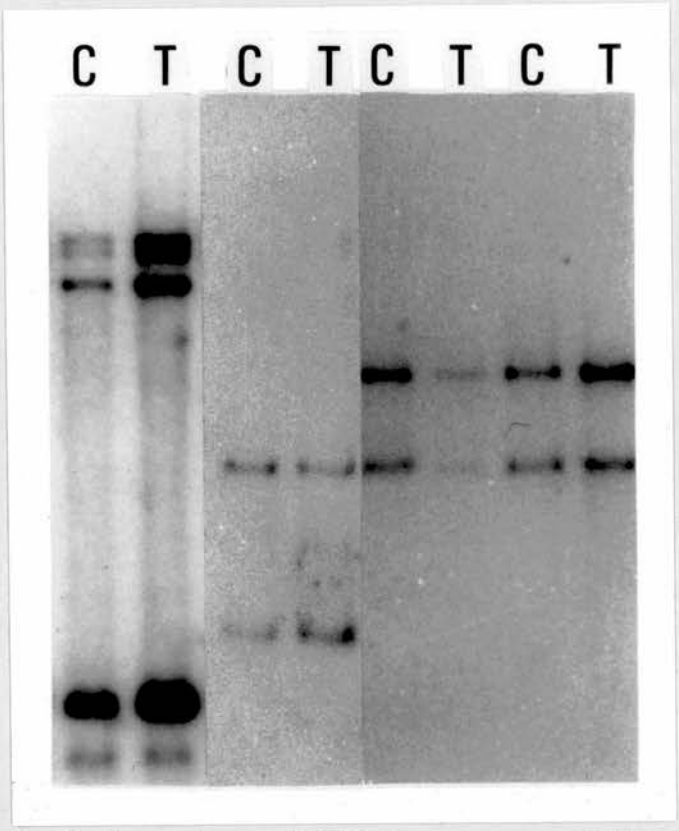


Fig 11 Paired digestions of DNA from tumour (T) and lymphoblastoid cell lines (C) from i) one patient probed with PGA (Eco R1) and ii) Three patients probed with λMS8 (Hinf 1). Note that none of the tumours have lost an allele.

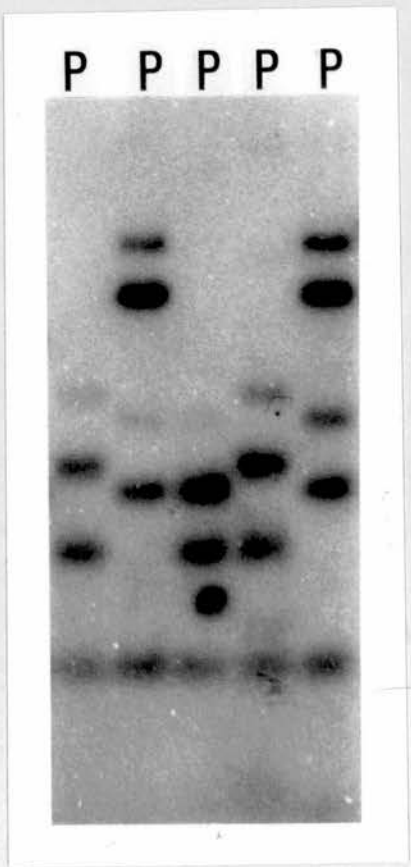


Fig 12 MspI digestions of DNA from 5 placental samples probed with YNZ 22 showing the complex binding pattern, although different genotypes can be recognised.

Loss of a YNZ 22 allele in tumour DNA

A series of fifty tumour/cell lymphoblastoid lines or tumour/white blood cell pairs was examined from forty-five individuals, three patients having two discrete tumours, and one having three. Twelve individuals were constitutionally homozygous, and of 38 heterozygous tumours there was allelic loss in 23 (61%). Two informative areas on 17q were also examined, as seen in the table below, demonstrating that the majority of tumours have not lost a whole copy of chromosome 17.

TABLE 7

Chromosomal location	Gene or probe	Restriction enzymes	No of Tumours examined	No of Patients heterozygous	No of tumours showing loss	
17p 13.3	YNZ 22.1	Taq 1	50	38	23 (61%)	
17q 21.3	Erb A2 (pHe A2)	Bam H1	20	11	0	3.3%
17q 25	pTHH59	Taq 1	35	19	1*	

*Uninformative for 17p.

In two instances separate (but coincident) tumours from the same patient differed in respect of YNZ 22 allele status (as seen in Fig 13).

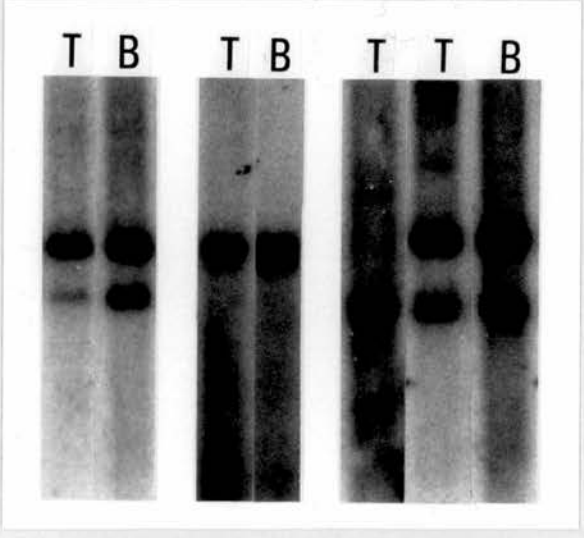


Fig 13 Taq 1 digestion of tumour (T) and leucocyte (B) DNA from 3 patients probed with YN2 22. The right hand trio shows allelic loss in one tumour, but probable retention in the other.

Of the fourteen tumours showing loss of a Harvey ras allele, ten (71%) had lost a YNZ 22 allele - evidence that the two events are neither mutually exclusive nor preferentially associated using the X^2 test on tumours informative for both loci.

In keeping with that observation, loss of a YNZ 22 allele was not correlated with low oestrogen receptor protein level, metastatic lymph node involvement or family history as tabulated below (the 12 uninformative individuals for YNZ 22 are excluded).

TABLE 8

Association between loss of a YNZ 22 allele and oestrogen receptor content

	ER +ve ($\leq$20fmol/mg)	ER -ve (>20fmol/mg)
YNZ 22 loss	8	13
YNZ 22 no loss	9	4

4 tumours excluded $X^2 = 3.24$ N.S.

TABLE 9

Association between loss of a YNZ 22 allele and metastatic lymph node involvement

	Node +ve	Node -ve
YNZ 22 loss	10	13
YNZ 22 no loss	9	6

All tumours included $X^2 = 0.76$ N.S.

TABLE 10

Association between loss of a YNZ 22 allele and family history (one or more first degree relatives affected with breast cancer)

	+ve Family History	No Family History
YNZ 22 loss	2	16
YNZ 22 no loss	3	10

Individuals with tumours with different genotypes excluded.
$X^2 = 1.06$ N.S.

There was no association between YNZ allelic loss and tumour size or mean age at presentation.

7 YNZ 22 ALLELES IN A LARGE KINDRED WITH FAMILIAL BREAST CANCER

A large kindred with a high incidence of breast cancer was identified and the pedigree extended and verified. The full pedigree is illustrated in Figure 14.

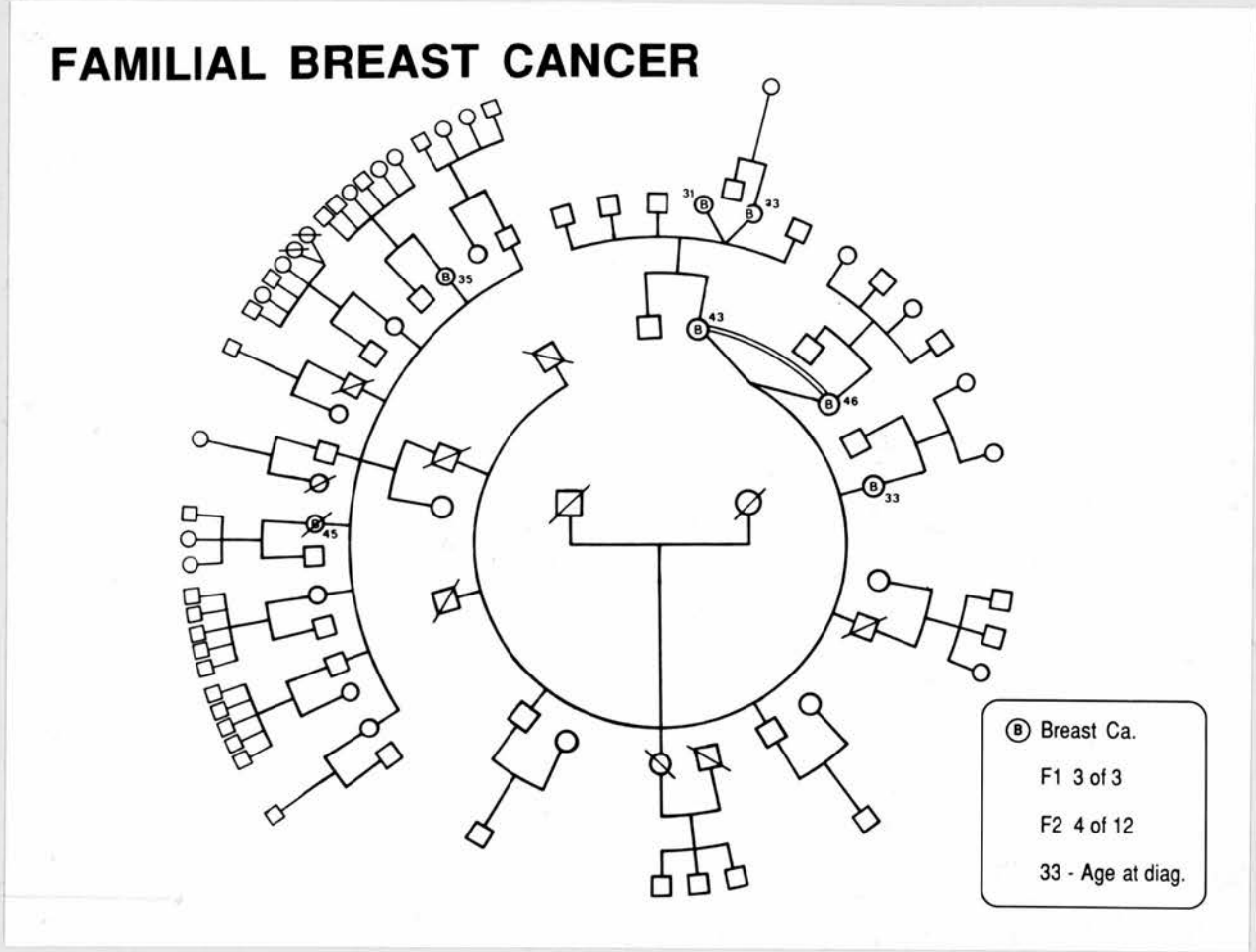


Fig 14 Pedigree of a family with a high incidence of breast cancer. Note 7 individuals with breast cancer out of 16 over the age of 30.

Of this kindred seven females had histologically proven breast cancer (3 bilateral) out of 16 at risk (discounting those under 30 years old) in 3 generations. Age at diagnosis ranged from 31 to 46 years. Inheritance followed a pattern consistent with an autosomal dominant trait with incomplete penetrance. There was no apparent excess of other cancers.

Among the affected individuals was a pair of monozygotic twins, and another pair of dizygotic twins. The Taq I YNZ 22 genotypes of both affected and unaffected individuals in this kindred, either directly ascertained or inferred are illustrated in Figure 15.

For this kindred, the following lod scores were calculated by Dr J F Clayton, using a programme derived from a MOLL/SITU package as previously described (Clayton 1986). Calculating these lod scores involved making assumptions about:

The mode of segregation of the disease gene, the local disease gene frequency, and the local disease gene penetrance, as made by Williams in a Danish complex segregation analysis (Williams et al 1984).

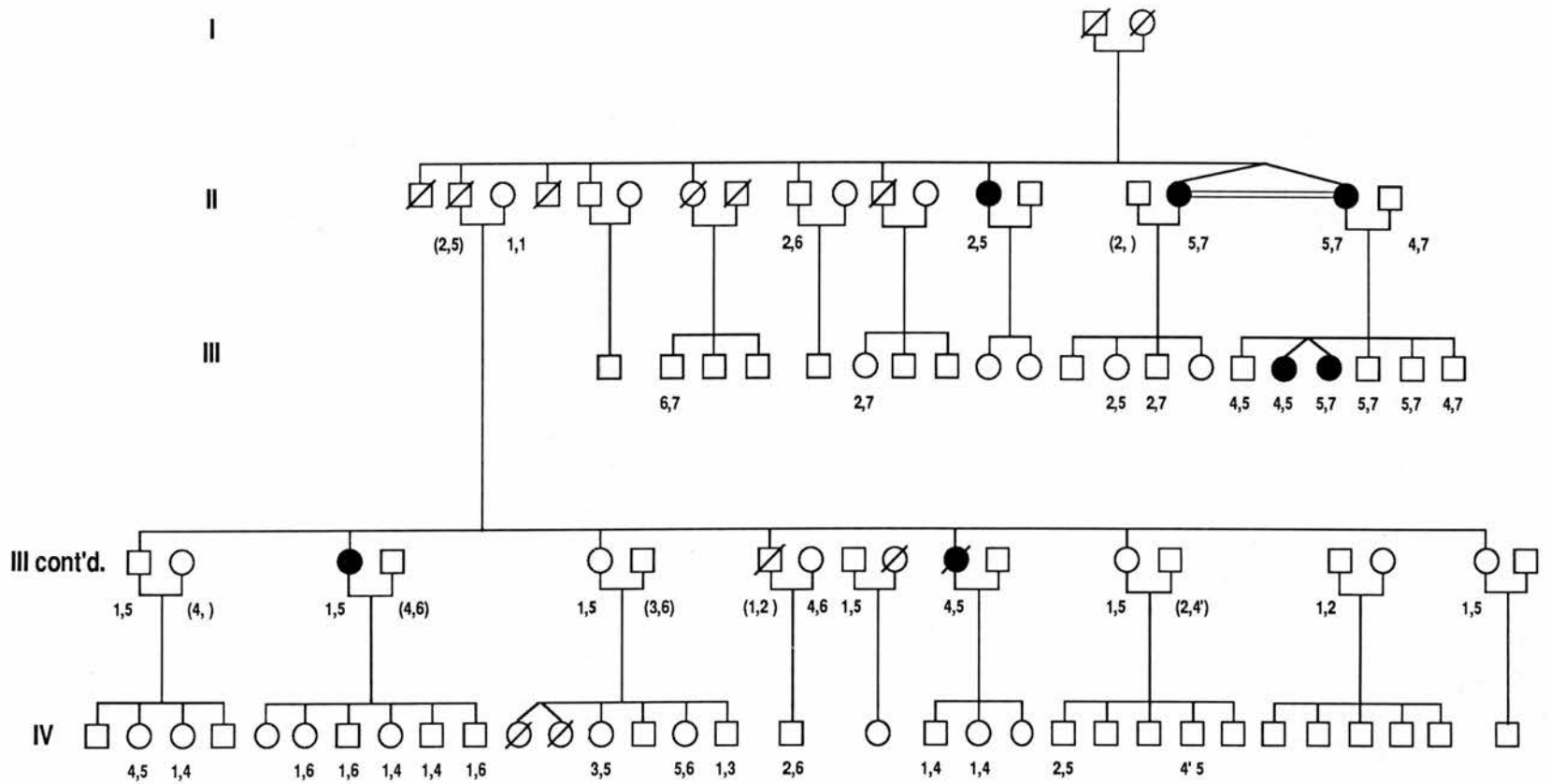
(1) Assume a very rare gene with very low penetrance:- lod score at 0 recombination = 1.8.

(2) Assume a rare gene with 60% penetrance in an 80 year old carrier:- lod score at 0 recombination = 1.06.

(3) Assume a fairly common gene with 60% penetrance in an 80 year old carrier:- lod score at 0 recombination =0.68.

While these results fall short of definitive proof that YNZ 22 alleles are linked to susceptibility to breast cancer, they are highly suggestive.

Fig 15 Taq I YNZ 22 genotypes of the important individuals in the family illustrated in Fig 14. Solid symbols represent individuals with breast cancer



DISCUSSION

Frequency of rare Bam Hl Harvey ras alleles

1 These results, in agreement with several published studies (Krontiris 1985; Thein 1986, Heighway 1986) demonstrate that rare Ha-ras alleles can be identified in the normal population. However, we have found no evidence for an increased frequency of rare alleles in breast cancer patients, contradicting both Krontiris' initial report (1985), and the findings of a subsequent larger series (Lidereau 1986) in which rare alleles were identified in 41% of breast cancer patients.

As mentioned in the introduction (p26), it was essential to substantiate or refute Krontiris' initial report, by performing large studies on patients with a single tumour type. Several of these have now been published, by other groups.

Thein (1986) reported rare alleles in 2.8% of patients with myelodysplastic syndrome compared to 4.8% in controls. Gerhard (1987) reported 4% of melanoma patients had rare alleles compared to 6% in controls, confirmed by Hayward et al (1988). Heighway (1986) reported 5-7% in lung cancers, compared to 4% in controls. Ceccherini-Nelli (1987) reported rare Taq I alleles in 4% of colonic adenocarcinoma patients compared to 2.5% in controls, supported by a carefully controlled study (Wyllie 1988) of Bam HI and Ava II alleles in colorectal cancer patients. This last study highlights the need for considering all sources of artefact in the Southern blots. The authors found that loading the correct weight of DNA in each slot was critical, and also noted that in several tumour DNA samples partial degradation of the DNA during storage resulted in the identification of apparently "rare" alleles. The studies reported by Lidereau et al (1986) in breast cancer patients were almost all performed on tumour DNA, from patients, whereas white blood cell DNA was used for

controls. Our carefully controlled study, initially reported in early 1987 (Mackay 1987), using placental markers on both sides of the gel, and comparing white blood cell DNA to tumour DNA from the same patient in any cases of doubt, as recommended by Wyllie et al (1988) support this interpretation, and unequivocally demonstrate that rare Harvey-ras alleles are not increased in breast cancer patients. This agrees with a subsequent, smaller study, reported by White (1988).

Wyllie's conclusions are further supported by Hayward's study (1988), as he looked at either white blood cell DNA or cell line DNA from patients and relatives with melanoma and Wilm's tumour. He also examined DNA from transitional cell bladder tumours and reported a significant percentage of rare alleles only in bladder carcinoma, the one group in which the analysis was performed on tumour tissue.

Radice (1987) digested white blood cell DNA from 55 melanoma patients, with Taq I. He identified an additional polymorphism, separate from that detected by Bam HI or MSP/HPA, which had been previously reported (Pierrotti 1986). This Taq 1 polymorphism was present in 18% of melanoma patients, significantly greater than in his control population (6%). In the absence of other studies examining this particular polymorphism the significance of his observation remains unclear.

Heighway originally reported (1986) a preponderance of the common Bam HI A4 allele in non-small cell carcinoma of the lung, and Wyllie (1988) reported a preponderance of the two larger common alleles A3 and A4 in colorectal cancer.

In agreement with White et al (1988) we found no significant difference in the frequency of any of the common alleles in breast cancer patients.

Harvey ras allelic loss in tumour DNA

Our finding of loss of a Harvey-ras allele in 14 of 65 informative tumours (21.5%), is in good agreement with the small study of Yokota (1986) and the larger study of Theillet (1986). The fact that there was no preferential loss of larger alleles, and that allelic loss was found in only 1 of 67 informative loci outside 11p, make it very unlikely that this finding was an artefact caused by degradation of the tumour DNA as mentioned by Wyllie (1988). The significant correlations between Harvey ras allelic loss and paucity of oestrogen receptor protein, and between Harvey-ras loss and larger tumours, contrasting with the absence of any significant correlation between Harvey-ras allelic loss and regional lymph node metastases, all agree with the findings of Theillet et al (1987). They concluded that Harvey-ras allelic loss was significantly linked to parameters of tumour aggressiveness on the basis of observed correlations between Harvey-ras allelic loss and paucity of oestrogen receptor protein, histological grade, and early occurrence of distant metastases. Their studies were performed on tumour samples stored for up to 7 years. As our analysis was done prospectively, we will have to wait for data on disease free interval and 5 year survival, before we can comment authoritatively on the prognostic importance of Harvey ras allele loss.

Our detailed data on 19 tumour/cell line pairs, fully characterised for 5 loci on 11p outwith the Harvey ras locus, presented in Table 5, suggest that it is likely to be a locus on 11p near Harvey-ras, rather than Harvey-ras itself which is of importance in breast cancer. These data are compatible with simple mitotic recombination below 11p13 in some tumours, though more complex mechanisms may be involved in other tumours. This refutes the smaller study of Ali et al (1987) who suggest the existence of a putative locus between the

beta-globin and PTH loci, which must be deleted en route to full-blown malignancy. This issue will only be fully resolved by a larger study, examining 11p in more detail, and making use of the growing number of highly polymorphic DNA sequences currently being characterised to study the WAGR locus (Porteous 1987; Boyd 1988).

However, there has clearly been a substantial frequency of DNA lesions within the short arm of chromosome 11 in our tumour material, lending support to the localisation of a putative tumour suppressor gene in breast cancer, to 11p (Mackay 1988a).

Involvement of 17p in familial and sporadic breast cancer

A note of caution must be sounded when drawing conclusions from genetic lesions found in tumour tissue. Biochemical and cytogenetic evidence has been available for many years to show that malignant cells tend to accumulate multiple aberrations in their DNA, including gene deletions (Povey 1980; Sandberg 1980). Recent studies using highly polymorphic probes of unknown locus specificity have confirmed these findings (Thein 1987), and allelic loss on 13q has been demonstrated in a small number of ductal breast cancers (Lundberg 1987). It is very unlikely that all these aberrations contribute to the malignant state.

The last part of our study neatly side-steps this problem, and provides definite experimental evidence supporting the theoretical strategy of attempting to identify DNA sequences consistently lost in tumour tissue, as outlined in the introduction (p8-10, 23,24).

Our finding that 61% of informative breast tumours have lost one Taq I YNZ 22 allele, coupled with the tight linkage demonstrated between the same locus and the trait of susceptibility to develop hereditary breast cancer in one large kindred, appears analogous to the findings in retinoblastoma outlined, at length, in the Introduction (p6-10).

These findings strongly suggest that 17p plays an important role in the aetiology of breast cancer. Accepting that carcinogenesis is a multi-stage process, we do not yet know whether 17p is involved in the early or late stages.

In two individuals, who had two discrete tumours, we have identified YNZ 22 allelic loss in only one of each pair, suggesting that 17p involvement is a "later" event which is in broad agreement with Fearon's conclusion that in colonic carcinoma (Fearon 1987), loss of sequences on 17p is a late event in the progression from adenoma to carcinoma. This is the first report of 17p deletions in sporadic breast cancer (Mackay 1988b) confirmed by Devilee et al (1989) but there have been no previous reports of linkage to 17p markers in familial breast cancer.

Our figure of YNZ 22 allelic loss in 61% of primary breast tumours might well be an underestimate, as several of the tumour DNA samples, showed a suspicious inequality in the intensity of the two autoradiographic bands, which although not scored as allelic loss, suggests that there may be a subpopulation of tumour cells that has undergone hemizygotisation, and that the true figure is closer to 76%, reported for colonic carcinoma (Fearon 1987).

We used YNZ 22 because although anonymous i.e. untranslated, it is highly polymorphic and therefore very informative (Nakamura 1988), but these findings do inevitably raise the question, "which gene on 17p is actually involved in breast cancer?"

One of the strongest candidates is p53, which has recently been mapped to 17p13, the same band as YNZ 22 (Van Tuinen 1988; Cohen-Haguenauer 1988). This gene has been fully discussed in the Introduction (p20-22), but a couple of facts bear repetition. It is expressed at a high level in 15% of aggressive primary breast tumours (Cattoretti 1988) and studies in the mouse have suggested that located upstream of a promoter sequence, there is a negative

regulatory sequence (Bienz-Tadmar 1985). It is quite possible, considering the other facts known about the activity of p53, that this negative regulatory element is, in fact, a "tumour suppressor gene". Obviously, further mapping studies are required to elucidate this point fully.

The significance and implications of 17p involvement

Our lack of knowledge means that several assumptions (e.g. local disease gene frequency, disease gene penetrance) have to be made in calculating the lod score. A lod score of 1.8 calculated by Dr J F Clayton, on the basis of Williams and Anderson's (1984) data, is encouragingly high for a preliminary study. Given these constraints, it is likely that statistical significance will only be reached by a lod score of well over 3. As explained in the introduction (p26-31), the next step is to examine several other families, with clear transmission of the susceptibility trait. This will inevitably involve collaboration with other large breast cancer units throughout the country.

If our preliminary findings are confirmed, we will then be able to screen relatives of patients with a strong family history of breast cancer. Because the disease gene clearly demonstrates incomplete penetrance, we would not be able to say definitively that any individual was going to develop breast cancer, but we would be able to identify individuals who were not at increased risk of developing hereditary breast cancer.

Hereditary, as opposed to sporadic, breast cancer, only comprises a small percentage (18%) (Lynch 1984), of the total pool of breast cancer, and so these findings are unlikely to make a large impact on population-based screening. They will allow us to identify a subpopulation of individuals, previously thought to be "at high risk", due to family history, who are in fact not at such high

risk, although of course, we will be unable to determine the risk of their developing sporadic breast cancer.

The second major implication of these findings, is that we now know the chromosomal localisation of a gene likely to be of importance in both breast and colonic carcinoma. The finding that loss of a region on 11p is not associated with loss of YNZ 22 on 17p is further evidence for the multi-stage nature of human carcinogenesis. Vogelstein (1988) has proposed a sequence of events in the genesis of colonic cancer and it is very likely that a similar sequence will be involved in the genesis of breast cancer. Molecular genetics is at the stage of identifying individual components of this multifactorial process, and this work has made a significant contribution to the identification of two of these components.

Further progress will only be made by pinpointing the actual genes involved on 11p and 17p, and understanding how they interact in the process of evolution and progression of malignancy.

This knowledge will almost certainly open up new opportunities for prevention and treatment of breast cancer.

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ALLELE LOSS ON SHORT ARM OF CHROMOSOME 17 IN BREAST CANCERS

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Summary Tumour and blood leucocyte DNA from a consecutive series of patients with primary breast cancer was probed to detect deletions at six polymorphic loci in tumour tissue. The highest frequency of allele loss (61%) was found with the probe YNZ22, which detects a sequence on the short arm of chromosome 17 (at p13.3). The previously reported loss of alleles at the Harvey ras locus (11p14) in about 20% of breast tumours was confirmed. The putative breast tumour suppressor gene on 17p may be the same as that already noted for colon and lung cancers and it is suggested that deletion of this gene is one of a cumulative series of lesions involving genetic changes in the evolution of breast cancer. The findings identify chromosome 17p as a candidate region for linkage studies in breast cancer families.

Introduction

THERE is growing recognition of the importance of suppressor genes ("anti-oncogenes") in human cancers.[1-4] These genes are recognised by their inactivation or loss (usually detected as deletion of the chromosome region bearing one copy) in a substantial proportion of tumours of a given histological type. The inactivation of a series of tumour suppressor genes and the activation of one or more oncogenes appear to be cumulative steps on the route to overt malignancy in the case of colon cancer,[5] and this model is probably valid for other human tumours.[6]

Heritable lesions affecting several tumour suppressor genes (some still putative) seem to determine genetic susceptibility to malignancies behaving as autosomal dominant disorders with variable penetrance. This pattern of inheritance is believed to underlie familial aggregations of breast cancer that have been recognised for over a century,[7] but as yet there are no data on the location of a predisposing gene or genes.[8-11] The present study was designed to locate tumour suppressor genes involved in sporadic breast cancer and which would be candidates for genetic linkage studies in familial cases.

Patients and Methods

Fresh tissue was obtained from 100 consecutive resectable primary breast tumours removed at operation from patients presenting to the breast cancer clinic of the Edinburgh University Department of Clinical Surgery. All tumours were less than 5 cm diameter at diagnosis and no patient had received radiotherapy or chemotherapy (other than the anti-oestrogen tamoxifen) before surgery. A blood sample was also obtained from each patient and DNA was extracted from both tumour tissue and blood leucocytes by conventional methods.[12] In many instances a lymphoblastoid cell line was established from the blood lymphocytes, by transformation with Epstein-Barr virus, to provide a renewable source of the patient's constitutional DNA.

10 μg samples of DNA from tumours, blood leucocytes, and lymphoblastoid cell lines were digested with each of the restriction enzymes listed in the table. The digests were electrophoresed through 0·8% agarose, transferred to nylon membranes (Hybond, Amersham), and probed with the seven sequences also listed in the table. Probes were previously labelled with ^{32}P dCTP or dTTP to a specific activity of at least 5×10^7 cpm/μg by nick translation.[13] Filters were washed at 65°C with 0·1 × SSC (15 mmol/l NaCl, 1·5 mmol/l sodium citrate, 0·1% sodium pyrophosphate, 0·1% sodium dodecylsulphate) and exposed to Kodak 'X-Ar' film at −70°C for 7–14 days with intensifying screens.

Not every tumour has been genotyped with all seven probes because the quantity of tumour DNA is limited and must be conserved for detailed mapping studies that will follow this type of screening.

Results

As shown in the table, there was little or no evidence of gene deletion in tumour tissue for four of the six chromosomal regions examined (5q34, 6p21, 11q12, and 17q21–25). We did confirm the previously reported loss of a Harvey ras allele (11p1.4) in about 20% of tumours[15,16] and this observation has been discussed in detail elsewhere.[17] The most striking finding, however, was that 23 of the 38 tumours from patients constitutionally heterozygous at the 17p locus defined by the probe YNZ22[18] had undergone partial or complete loss of one allele (figure). That this loss is confined to a region of the short arm of chromosome 17 and does not reflect loss of a complete chromosome is evident from the retention of heterozygosity at 17q in those tumours informative for pHeA2 and PTHH59. In two instances separate (but coincident) tumours from the same patient differed in respect of YNZ22 allele status (figure). Several of the tumour DNA samples that were scored as having no allele loss nevertheless showed a suspicious inequality in the intensity of the two YNZ22 Taq 1 autoradiographic bands, suggesting the presence in the tumour mass of a population of cells that had indeed undergone hemizygotisation; so the figure of 61% of primary breast tumours showing involvement of a 17p locus is almost certainly an underestimate. Of the fourteen tumours showing loss of a c-Ha-ras allele (11p) ten (71%) had also lost a YNZ22 allele—evidence that the two events are neither mutually exclusive nor preferentially associated.

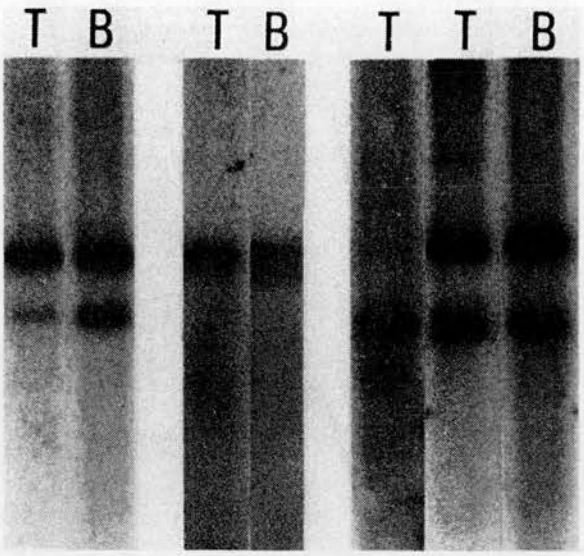
In keeping with that observation, we found that loss of a YNZ 22 allele was not correlated with such adverse prognostic indices as low oestrogen receptor level or

NUMBERS OF BREAST CANCERS SHOWING SPECIFIC ALLELE LOSS IN PATIENTS CONSTITUTIONALLY HETEROZYGOUS FOR VARIOUS DNA MARKERS

Chromosome	Gene or probe (ref 14)	Restr enzyme	No of tumours examined	No of patients heterozygous	No of tumours showing loss
5q34	λMS8 (D5S43)	HinfI	18	15	1 (7%)
6p21.3	MHC11 (p11-B-4)	EcoRI	21	21	0
11p1.4	Ha-ras (pEj)	BamH1	100	65	14 (21%)
11q1.2	Pepsinogen (pH PEP)	EcoRI	24	20	0
17p13.3	YNZ22.1	TaqI	50	38	23 (61%)
17q21.3	Erb A2 (pHeA2	BamH1	20	11	0 } (3·3%)
17q25	pTHH59	TaqI	35	19	1* }

*Uninformative for 17p.

The DNA probes listed were generously supplied by the following: λMS$_8$, Prof Alec Jeffreys; P11B4, Dr Dan Larhammar; pEj, Dr Chiah Shih; pHPEP, Dr R Thomas Taggart; YNZ 22 and pTHH 59, Dr Yusuke Nakamura; and pHeA2, Dr Matts Jansson.



Audioradiograph of tumour (T) and blood leucocyte (B) DNA from three breast cancer patients, digested with Taq 1 and Southern blotted with labelled probe YNZ22.

Tumour tissue from the patient in the left hand panel shows a marked reduction in intensity of the lower allele, indicating either that the tumour is a mosaic of heterozygous and hemizygous malignant cells or that there is a substantial admixture of heterozygous non-malignant cells (stroma, infiltrating lymphocytes, &c) in a hemizygous tumour. The tumour sample in the centre panel shows complete absence of one YNZ22 allele while the patient whose samples are shown in the right hand panel had two discrete primary tumours, one of which retained both YNZ22 alleles, the other being hemizygous. Note that, as expected for a polymorphic system, some allelic bands are common to two or more patients. In the material studied there is no apparent tendency for loss of any particular allele.

increased size of tumour at presentation, whereas loss of a c-Ha-ras allele correlated with both.[17]

Discussion

This is the first report of an association between loss of sequences on chromosome 17p and breast cancer. Involvement of this region has, however, been described in colorectal carcinoma,[5,19,20] in lung cancer,[21] and in osteosarcoma.[22] In the case of colon cancer, there is evidence for cumulative genetic lesions leading to overt malignancy.[5] These include inactivation of putative tumour suppressor genes on 5q, 17p, and 18q and activation of one of the ras family of oncogenes. Separate studies of lung cancer have identified probable tumour suppressor genes on 3p, 13q, and 17p while in breast tumours there is now substantial evidence to implicate corresponding sequences on 11p, 13q, and 17p as well as amplification of the Erb B2/neu oncogene.[15-17,23-25] In addition, cytogenetic analyses of near-diploid primary breast tumour cells have revealed a variety of chromosome deletions, translocations, and complete losses with particularly frequent involvement of chromosome 1 and the long arm of chromosome 11 at q13.[26-28] All of these findings are consistent with the substantial body of evidence indicating that an accumulation of genetic events is required for the evolution of frank malignancy. Our observations of disparity in terms of 17p allele loss between two breast tumours occurring simultaneously in the same patient may imply that loss of the putative tumour suppressor gene at that locus need not be an early event in the evolution of breast cancer, but it should also be borne in mind that the crucial deletion or inactivation of a gene on 17p must be close to, but may not always include, the YNZ 22 locus. Heritable cancer syndromes behaving as autosomal dominant disorders have been shown to be associated with lesions on 3p (renal cell carcinoma), 5q (familial adenomatous polyposis), 10 (multiple endocrine neoplasia type 2), 11q (multiple endocrine neoplasia type 1), 11p (Wilms' tumour), and 13q (retinoblastoma),[4] though only in the case of the 13q locus is there direct evidence that precisely the same DNA sequence is implicated in the inherited condition and in sporadic lung and breast tumours.[23,24,29] The present findings identify the short arm of chromosome 17 as a candidate region for localisation of a gene conferring increased susceptibility to breast cancer. The fact that the same region has also been implicated in sporadic tumours of colon and lung is of particular interest in view of the extensive evidence that "breast cancer families" commonly show, in addition, an excess of other types of tumour.[7,10,11]

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Partial deletion of chromosome 11p in breast cancer correlates with size of primary tumour and oestrogen receptor level

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Summary In a study of DNAs from 100 breast cancer patients and 100 controls, there were no differences in the frequencies of common or rare alleles at the Harvey *ras* (c-Ha-*ras*) locus on chromosome 11. However, one Ha-*ras* allele was deleted from the tumour DNA in 14 of 65 informative patients. Loss of a Ha-*ras* allele correlates with paucity of oestrogen receptor protein and with increased tumour size at presentation, but is not associated with microscopic evidence of lymph node invasion. The findings on Ha-*ras* and other informative loci are consistent with the possibility that a tumour suppressor gene involved in the early stages of breast cancer is located on the short arm of chromosome 11.

The human Ha-*ras* oncogene, homologous to the transforming sequence of the Harvey murine sarcoma virus, has been assigned to the short arm of chromosome 11 (McBride *et al.*, 1982). On the 3′ side of the gene lies a non-coding region made up of a variable number of repeated sub-units (Capon *et al.*, 1983). Digestion with the restriction enzyme Bam HI generates a restriction fragment length polymorphism with 4 common and several rarer alleles. Krontiris *et al.* (1985) have reported an increased frequency of rare alleles in patients with a variety of solid tumours and haematological malignancies; their series included a small number of breast cancers. This finding would imply that an inherited predisposition to cancer is linked to alleles at the Ha-*ras* locus. Several subsequent studies have sought to test Krontiris' hypothesis as applied to lung cancer (Heighway *et al.*, 1986), myelodysplasia (Thein *et al.*, 1986), colonic adenocarcinoma (Ceccherini-Nelli *et al.*, 1987), familial melanoma (Gerhard *et al.*, 1987; Hayward *et al.*, 1988) and breast cancer (Lidereau *et al.*, 1986). Only in the last two of these has supporting evidence been forthcoming (Lidereau *et al.*, 1986; Hayward *et al.*, 1988). However one group has recorded that a proportion of breast tumours, from patients constitutionally heterozygous at the Ha-*ras* locus, express only one allele or show a marked disparity in the intensity of the two allelic bands, suggesting that most or all of the tumour cells have undergone loss of a part of chromosome 11p (Theillet *et al.*, 1986; Ali *et al.*, 1987). Reduction to homo- (or hemi-) zygosity at specific genetic loci was recognised initially in retinoblastoma and subsequently in several other tumours (Knudson, 1971; 1985). The loci involved are believed to be sites of tumour suppressor genes or 'anti-oncogenes' which are relevant both to somatic events giving rise to sporadic tumours and to genetic predisposition to cancers (Lancet, 1988). In view of the potential importance of these issues for breast cancer screening programmes we have undertaken a survey of Ha-*ras* alleles in a cohort of 100 breast cancer patients.

Patients and methods

Tumour and venous blood samples have been collected from 100 consecutive patients with histologically proven breast cancer, prior to any treatment (apart from the anti-oestrogen tamoxifen). All patients had presented with palpable breast lumps and were referred by their general practitioners to the breast clinic in the Royal Infirmary of Edinburgh. Patients with T4 tumours or with distant metastases at presentation, were excluded, as they are usually treated by chemotherapy in the first instance. The surgical procedures performed were either modified Patey mastectomy with axillary clearance, or wide local excision with axillary lymph node sampling. The resected specimen was immediately examined by the pathologist, tumour diameter measured in mm, and blocks taken for histological examination and for oestrogen receptor protein assay. The remainder was frozen on dry ice for later DNA extraction.

Lymph nodes were processed and examined for microscopic metastatic invasion. Tumours were classified into histological types as previously reported (Page & Anderson, 1988). Oestrogen receptor concentration was determined immediately by a saturation analytical method with separation of free and bound hormone using Dextran-coated charcoal adsorption as previously described (Hawkins *et al.*, 1981). Samples from patients who had received Tamoxifen were rechecked by enzyme immunoassay (Leclercq *et al.*, 1986). One hundred fresh placental samples have also been collected to act as a panel of normal controls representative of the local Edinburgh population. Permanent lymphoid cell lines were established from many of the blood samples by transformation *in vitro* with Epstein Barr virus. DNA was extracted from tumour and placental tissues and from blood and lymphoid cell lines (Steel, 1984). Ten μg aliquots of genomic DNA were digested to completion with Bam HI (Roberts *et al.*, 1977) (Boehringer, Mannheim GmbH), electrophoresed through 0.8% agarose, transferred to nylon membranes (Hybond, Amersham) and hybridised according to the manufacturer's instructions with the Harvey *ras* probe pEj (Shih & Weinberg, 1982), nick translated to a specific activity of $5 \times 10^7 - 1 \times 10^8$ cpm μg^{-1} (Rigby *et al.*, 1977). After hybridisation, filters were washed at 65°C with 0.1XSSC (15 mM Na Cl, 1.5 mM NA_3 citrate, 0.1% Na PPi, 0.1% SDS) and exposed to Kodak X-Ar film at −70°C for 7–14 days, with intensifying screens. Similar procedures were followed with other probes and restriction enzymes as tabulated below.

Results

Allelic frequency

The four major c-Ha-*ras* Bam HI alleles A_1–A_4, together with one rare variant A'_1 are shown in Figure 1.

Table I shows the relative frequencies of these alleles in blood and/or lymphoid cell line DNA from 100 breast cancer patients and in DNA from 100 placentae.

There was no significant difference between breast cancer

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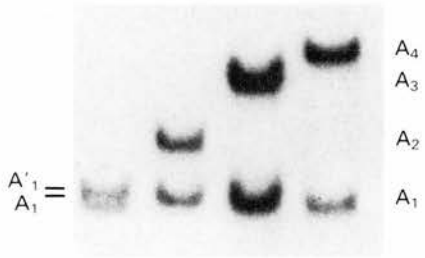


Figure 1 Alleles of c-Ha-*ras* (Bam HI digests) from four placental DNA samples. The left hand track of this Southern Blot contains a 'doublet' of allele A_1 and the rare variant A'_1.

Table I Bam HI alleles of Harvey *ras* locus

	100 Breast cancer patients		*100 Placentae*	
	Number	*%*	*Number*	*%*
A_1	126	63.0	135	67.5
A_2	25	12.5	27	13.5
A_3	23	11.5	19	9.5
A_4	19	9.5	15	7.5
Rare alleles[a]	7	3.5	4	2.0

[a]A'_1 and A''_1 . . . slightly smaller and larger respectively than A_1.

patients and controls in the frequencies of rare Harvey *ras* alleles; nor was there any shift in the distribution of common alleles between the two groups.

Allele loss in tumours

Complete or partial loss of a c-Ha-*ras* allele was established by comparing paired tumour DNA and white blood cell DNA samples from the same patients (Figure 2).

The one hundred tumours analysed fall into the following three categories. No allelic loss at the Ha-*ras* locus (51 tumours), loss of one allele (14 tumours) and uninformative, because the patient was constitutionally homozygous (35 tumours).

There was no preferential loss or retention of any of the four common alleles and our present analysis does not allow us to determine the maternal or paternal derivation of a deleted allele. We found no significant correlation between allelic loss and menopausal status, age, or history of an affected first degree relative. However, as shown in Table II, there was a significant correlation between loss of a Ha-*ras* allele and paucity of oestrogen receptor protein; absence of oestrogen receptor being a well-recognised index of poor prognosis (Croton *et al.*, 1981; Moore *et al.*, 1983; Williams *et al.*, 1987).

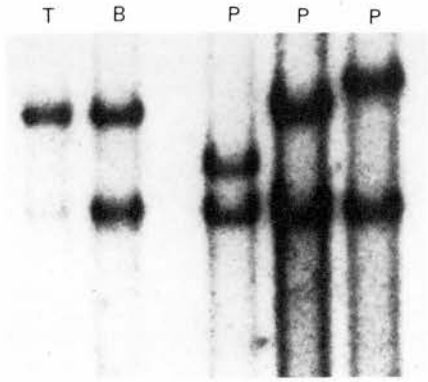


Figure 2 Bam HI digests of tumour (T) and blood leukocyte (B) DNA from the same patient, probed with c-Ha-*ras* and compared with three placental controls. Note that alleles A_1 and A_3 are of equal intensity in B but allele A_1 is almost absent from the tumour sample.

There was also a significant correlation between tumour size and allelic loss as shown in Figure 3.

There was no significant correlation between allelic loss and pathological lymph node involvement, vascular invasion or histological type of tumour.

In order to assess the specificity of loss of the Harvey *ras* allele we have examined up to 5 other loci on the short arm of chromosome 11, comparing tumour DNA with lymphoblastoid cell line DNA from the same patient, as detailed in Table III.

Heterozygosity was found on a total of 49 occasions and the corresponding tumours had lost an allele in 19 cases (38.8%). Nineteen tumour/cell line pairs have been fully characterised for all 5 loci and allelic loss at one or more has been found in 10 (53%).

We have also studied one informative locus (pepsinogen) on the long arm of chromosome 11 and three at other chromosomal sites (5q, 6p and 17q). Of 67 instances where the patient was constitutionally heterozygous allele loss in the tumour was found outside of the 11p region on only one occasion (Table IV).

Discussion

These results, in agreement with several published studies (Krontiris *et al.*, 1985; Heighway *et al.*, 1986; Thein *et al.*, 1986; Ceccherini-Nelli *et al.*, 1987; Gerhard *et al.*, 1987) demonstrate that rare Ha-*ras* alleles can be identified in the normal population. We have found no evidence for an increased frequency of rare alleles in breast cancer patients, contradicting both Krontiris' initial report (Krontiris *et al.*, 1985) and the findings of a subsequent larger series (Lidereau *et al.*, 1986) in which rare alleles were identified in 41% of breast cancer patients. Heighway *et al.* (1986) reported a preponderance of the A4 allele in patients with non-small-cell lung carcinoma, but several other studies have failed to find evidence of linkage in myelodysplasia (Thein *et al.*, 1986), colorectal adenocarcinoma (Hayward *et al.*, 1988) or familial melanoma (Gerhard, 1987). Lidereau's study on breast cancer patients was performed on breast tumour material which had been stored for up to 7 years, while the controls were fresh blood samples from unaffected individuals. Wyllie *et al.* (1988) has suggested that prolonged storage could lead to the identification of spurious 'rare' alleles, and we have therefore used DNA from white blood cells or lymphoblastoid cell lines, as well as tumour material.

In contrast to these negative findings, the observation that a substantial proportion of breast cancers have lost one c-Ha-*ras* allele confirms the recent report of Theillet *et al.* (1986) and lends some support to the hypothesis that the Ha-*ras* locus may be involved in breast cancer, albeit on a rather different theoretical basis.

Knudson's 'two hit' hypothesis (Knudson, 1971) provides a link between the molecular mechanisms underlying familial and sporadic forms of the same type of cancer. In sporadic cancer, a cell undergoes a somatic mutation, which must then be followed by a second event to express the malignant phenotype, either a second somatic mutation or loss of the unmutated allele by non-disjunction or deletion.

Following the localisation of the retinoblastoma gene to 13q14 (Cavenee *et al.*, 1983) comparable deletions at other sites have been reported in a variety of tumours, including Wilms' tumour (Koufos *et al.*, 1985), lung cancer (Kok *et al.*, 1987) and acoustic neuroma/meningioma (Seizinger *et al.*, 1986).

The present findings raise the question 'Is the reduction to homozygosity of the Harvey *ras* gene in breast cancer merely an indication that there has been a deletion somewhere on chromosome 11 and is there another gene in the region much more directly involved in the disease?' Ali and colleagues (1987) recently reported a total of 14 allele losses, distributed between five polymorphic loci on 11p in breast

Table II Relationship between loss of a c-Ha-*ras* allele and oestrogen receptor level in 61 breast tumours

	ER poor/-ve $<20 fmol\,mg^{-1}$ *protein*	*ER moderate/rich* $\geqslant 20 fmol\,mg^{-1}$ *protein (20)*
Allelic loss	8	6
No allelic loss	10	37

$P = <0.02$.

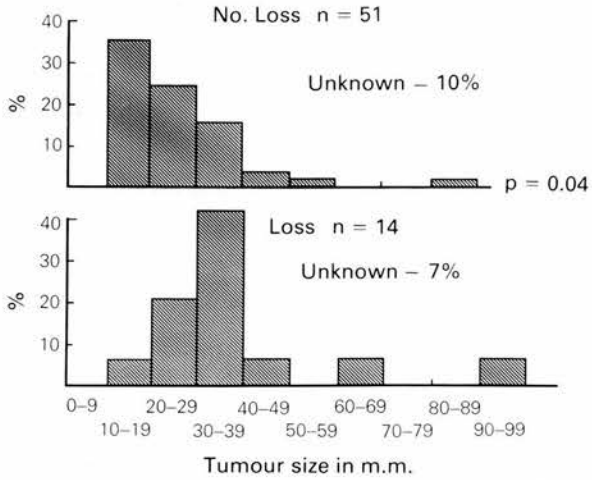
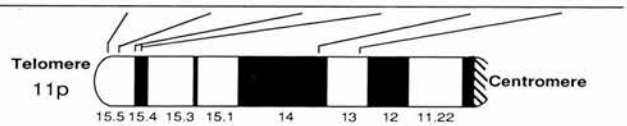


Figure 3 Distribution of primary tumour size (greatest diameter mm) in relation to Ha-*ras* allele status.

cancers from 9 patients, not all of whom were analysed for every locus. We find reduction to hemizygosity of several sequences other than Harvey *ras* on the same chromosome arm, at least one locus being involved in 10 of 19 tumours (53%), a frequency even higher than the corresponding figure for Ha-*ras* (21.5%) and certainly much higher than for informative loci outside 11p (1 of 67 informative loci, in 27 breast tumours). It might be unwise to extrapolate from the present data, for example, to suggest that 11p deletions can be inferred in almost 50% of primary breast cancers since only 19 tumours have been analysed in detail so far and they include 8 already known to have lost a Ha-*ras* allele. Nevertheless there has clearly been a substantial frequency of DNA lesions within the short arm of chromosome 11 in our tumour material. The simplest interpretation is that a single mitotic recombination event has caused loss of all loci distal to the breakpoint which, in some instances, must have been on the centromeric side of the most proximal sequence examined, P11F9, at 11p13. At least three of the tumours studied, however, show patterns of allele loss incompatible with this simple mechanism since one or more loci on the telomeric side of the region of hemizygotisation remain heterozygous and in one case (No. 8, Table III) there were two regions of hemizygotisation separated by a locus that remains heterozygous. It is necessary, therefore, to invoke either multiple mitotic recombination events, localised chromosome deletions, partial inversions or even more complex rearrangements. More extensive mapping studies are required to resolve the issues raised by these observations and analysis is now being extended to cover all one hundred tumours in our series. One objective is to identify the smallest region of chromosome 11p that is consistently included in any deletion that can be mapped. Such a region might then be the site of a putative tumour suppressor gene (Friend *et al.*, 1988).

Table III Details of allelic losses on 11p in breast tumour DNA compared with corresponding lymphoid cell line DNA

Tumour/ cell line pair	*Ha-*ras **1*	*β-globin *2*	*PTH *3*	*Calcit *4*	*FSH-β *5,6*	*P11F9 *7,8*
1	a–	u	a–	a–	a–	a–
2	a–	a–	u	u	a–	u
3	a–	u	u	a–	u	u
4	a–	u	u	ab	ab	u
5	a–	u	u	u	u	ab
6	a–	u	u	u	u	ab
7	a–	ab	u	u	u	ab
8	a–	ab	a–	u	a–	ab
9	ab	u	u	u	u	a–
10	ab	u	u	u	u	ab
11	ab	ab	ab	u	u	ab
12	ab	ab	ab	u	u	ab
13	u	ab	u	u	ab	a–
14	u	ab	a–	u	u	a–
15	u	u	a–	u	u	a–
16	u	ab	u	u	u	ab
17	u	u	ab	u	ab	u
18	u	a–	u	u	u	ab
19	u	a–	u	u	u	ab



ab = informative, not lost; u = uninformative; a– = allele loss.
'a' and 'b' should not be taken to refer to specific alleles.
*1 = Shih & Weinberg (1982); *2 = Deisseroth *et al.* (1978); *3 = Naylor *et al.* (1982); *4 = Höppener *et al.* (1984); *5,6 = Glaser *et al.* (1985), Watkins *et al.* (1985); *7,8 = Porteous *et al.* (1987), Boyd *et al.* (submitted).

This of course does not preclude the specific involvement of other regions of the genome not examined in the present study and it is quite possible that two or more putative anti-oncogenes are involved in breast cancer (Lancet editorial, 1988). It is relevant to note that loss of heterozygosity has been reported on 13q in 6 out of 10 ductal breast cancers (Lundberg *et al.*, 1987).

We find that loss of a Ha-*ras* allele correlates with paucity of oestrogen receptor protein and with larger tumour size at presentation, but there is no correlation between pathological lymph node involvement and loss of a Ha-*ras* allele. Theillet *et al.* (1986) concluded that Ha-*ras* allelic loss was significantly linked to parameters of tumour aggressiveness since, in their material there were correlations between allelic loss and paucity of oestrogen receptor protein, histological grade and early occurrence of distant metastasis.

Table IV

Gene or probe	*Designation*	*Localisation*	*Restriction enz*	*Ref*[a]	*No. examined*	*No. informative*	*No lost*
Pepsinogen	pH PEP	11q 12	EcoR I	1,2	24	20	0
Erb A_2	pHeA2	17q 21.3	Bam HI	3	20	11	0
MHC Class II	p11-B-4	6p 21.3	EcoR I	4	21	21	0
λ MS 8	D8 S43	5q 34qter	Hin FI	5,6	18	15	1
					Total	67	1

[a] 1,2 = Taggart *et al.* (1985; 1987); 3 = Gosden *et al.* (1986); 4 = Gustafsson *et al.* (1984); 5,6 = Solomon *et al.* (1987), Wong *et al.* (1987).

Of the six breast tumours identified in this series as uninformative or heterozygous for c-Ha-*ras*, but showing loss of an allele at one or more loci elsewhere on 11p (cases 9, 13, 14, 15, 18 and 19, Table III), five had low or absent oestrogen receptor. The correlation between this prognostic feature and hemizygotisation of sequences somewhere on 11p therefore does not seem to be exclusive to the Harvey-*ras* locus. Although such a conclusion must be tentative until a larger number of tumours has been analysed in similar detail, the suggestion is that c-Ha-*ras* serves as a relatively inefficient index of hemizygotisation of a specific locus some distance away.

It will be of great importance to map that putative locus and thereafter to reassess the clinico-pathological correlations already established, to see if information of prognostic value can be obtained from DNA analysis of tumours at presentation. The clinical relevance of such findings will be established by follow up of our patient cohort to gather data on disease-free interval and long-term survival. Ultimately the objective is to define the gene itself and to establish the mechanism whereby it contributes to the evolution of breast cancer.

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From

HIGH RISK BREAST CANCER.

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Genetic Aspects of Human Breast Cancer

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Risk Factors in General

In common with many neoplasms, the incidence of breast cancer increases with age [24]. It is slightly higher in socioeconomic classes I and II [52] and in North American and European women compared with African and Asian women [127]. The behaviour of the ovary appears to play a central role in the aetiology of breast cancer; several studies showing that early age of menarche (below 14 years) and late age of menopause, increase the risk [20, 107, 12, 119]. The effects of pregnancy are less clear; childbearing appears protective if the first child is born before the mother reaches the age of 30, although bearing more than five children increases the risk [12, 26, 125, 76, 1]. Alterations of the hormonal environment, as in prolonged use of oral contraceptives or postmenopausal oestrogens, do not appear to affect the risk in most recent studies, but this remains a controversial issue [12, 51, 122, 123, 124, 98, 78]. Artificial menopause, for example by oophorectomy, is reported to be protective; the earlier it is performed the greater the reduction in risk [69, 33].

Past medical history is of particular importance in the assessment of risk. A previous diagnosis of cancer in one breast significantly increases the likelihood of developing a second primary tumour in the other [66, 110]. Many studies have suggested that a history of benign breast disease, particularly if a biopsy was performed, increases risk [25, 77, 48] although this has been disputed [12, 18] and certainly the pathological findings in the biopsy are not predictive [78]. A history of primary cancer in the ovary or endometrium has also been reported to increase the chance of subsequent breast cancer [75, 92].

Many other factors, such as body mass, have been shown to be related to very small increases in risk [65, 61], but the strongest predictive factor so far identified and the one which we will now examine in more detail, is a positive family history of breast cancer.

Family History

The occurrence of families containing a large number of individuals affected by breast cancer has been recognised for many centuries. The first detailed report was published in 1866 by the French surgeon Paul Broca [13] who ascertained the

cause of death in 38 individuals through five generations of his wife's family. Of the 24 women in that family, 10 died of breast cancer and several more individuals died from other malignancies. Both Broca and his contemporary Sir James Paget [84, 63] expressed concern that multiple instances of such a common disease might appear in a small number of families by coincidence, but, using the available data on cancer mortality rates in the normal population, they concluded that a tendency to develop breast cancer could indeed be inherited.

Advances in statistics, epidemiology and genetics allowed more rigorous examination of these initial observations in the first half of the twentieth century. Several groups attempted to compare the mortality from breast cancer in a population of patients with one or more affected relatives and in a normal control population [49, 132, 4, 52, 70]. These studies highlighted the importance of pathological verification of malignancy, of assessing the incidence of malignancies other than breast cancer and of using large numbers of families plus reliable data on cancer incidence and mortality in the general population. Overall, they showed that there was a twofold increase in breast cancer incidence in the close female relatives of breast cancer patients. Underreporting of disease by control subjects was recognised as a significant problem which remains hard to surmount. Further analysis of data collected in the course of these studies hinted at the possibility of there being two separate types of breast cancer, sporadic and hereditary, and suggested that several features were commoner in one type than the other.

The important characteristics of familial breast cancer in a number of countries have been defined by H.T. Lynch and colleagues [71]:

1. Significantly early age of onset
2. Excess of bilaterality
3. Excess of multiple primaries at various sites
4. Vertical transmission (mother to daughter)
5. Impaired survival when compared with sporadic forms. Applying these criteria, he estimates that 5% of *all* breast cancer is familial, but 11.5% of patients with breast cancer diagnosed before the age of 50 have a familial form of the disease.

Anderson [5, 6] adopted a somewhat different approach, dividing his cases into subgroups and identifying the groups in which the risk was increased beyond the two- or threefold level observed in the earlier studies. The relative risk to first-degree female relatives of patients with premenopausal breast cancer was 3.1, while no increase in risk was observed among relatives of postmenopausal patients. If the patient had bilateral breast cancer the risk to first-degree female relatives was increased fivefold. If both conditions applied (i.e. the patient was premenopausal *and* had bilateral disease), the risk to first-degree relatives was increased ninefold. Furthermore, the relatives of patients with bilateral disease were at a ninefold times greater risk of developing bilateral disease themselves as compared with relatives of patients with unilateral disease.

The importance of age and of bilaterality have been confirmed in other studies [7, 17] and so, despite one dissenting report from a large Swedish study in which the effects of family history, age of onset and bilaterality were rather weak [2], we are now in a position to identify a group of women who are at significantly higher risk

of developing breast cancer than the general population, who are likely to be more aware of (and concerned about) their susceptibility to the disease [50], and who therefore require detailed and accurate counselling about that risk [83]. By combining complex statistical analyses and laboratory investigation it is possible to define more precisely the genetic component in what is acknowledged to be a disease of multifactorial aetiology.

Segregation Analysis

In some "classical" genetic disorders, inspection of the pedigrees of a few affected families will reveal the mode of transmission (autosomal or sex-linked, dominant or recessive). However, in the vast majority of disease, where the genetic component is less clear cut, a more comprehensive statistical analysis is required. Segregation analysis is the name given to the process of determining the probable mode of transmission of a trait, from an observed distribution of phenotypes in a pedigree or a number of pedigrees.

The procedure involves calculating how well the observed distribution of phenotypes fits various hypotheses and can thus establish the validity of some of these hypotheses, though it may not prove conclusively that a trait *is* transmitted genetically. The larger the families examined, the more affected individuals in each family; the larger the total number of families, the better will be the data and the more secure the final conclusions.

Several statistical advances, such as maximum likelihood scoring, the concept of multifactorial inheritance, the "mixed model" and a sampling correction to allow for the manner in which the pedigrees have been ascertained and relatives added [29, 30, 80, 27, 60, 81], have all been incorporated into a segregation analysis performed on 200 Danish families with breast cancer [131].

The observed distribution of breast cancer in these families was compatible with transmission of a single autosomal gene with dominant expression, the frequency of the abnormal (disease) allele being 0.7%, and the penetrance varying with age. According to that model, by age 80, a female heterozygous for the abnormal allele would have a 57% chance of developing breast cancer. For cancers presenting before age 30, 88% of affected females would be carriers of the disease gene whereas for the total population (presentation up to age 80), only 13% of affected females would carry the gene, the other 87% having developed "sporadic" breast cancer.

Several similar analyses have been performed on another large family with breast cancer and all agree that an autosomal dominant gene with incomplete penetrance is the most likely mode of transmission [35, 44, 36]. Increasing the complexity of a segregation analysis may well improve the accuracy of estimates of gene frequency and penetrance, but it cannot give any indication as to what gene is involved and where in the human genome it may be.

The Principles of Linkage Analysis

The most practical approach to locating the gene for susceptibility to breast cancer is by the technique of linkage analysis, based on the segregation of defined genetic markers in affected and unaffected family members [133]. The human genome is composed of genes arranged in a linear fashion along the 23 pairs of chromosomes. Genes which are close together on the same chromosome tend to be transmitted together, i.e. to segregate nonindependently. Genes on different chromosomes segregate independently so that every possible combination of alleles appears with equal frequency in the gametes, as illustrated by Fig. 1. The disease gene is D (normal allele d) and the marker gene alleles are represented by T and t.

If the disease gene and the marker gene are physically very close together, they will be transmitted together, so that the gametes formed are either DT or dt as shown in Fig. 2. This departure from independent segregation is termed "linkage", with D and T being very tightly linked in the example shown. If D and T are slightly further apart on the chromosome, the two genes may well be transmitted together, but because they can segregate by crossing over and recombination at meiosis, a few gametes with genotypes Dt or dT will appear, as illustrated in Fig. 3. These gametes are known as recombinants and the proportion of recombinant gametes in the total pool is the recombination fraction. The further apart the two genes are, the more likely it is that recombination will occur, until eventually the two genes will appear to be segregating independently as all four possible genotypes will be represented equally, giving a recombinant fraction of 0.5, as illustrated in Fig. 4.

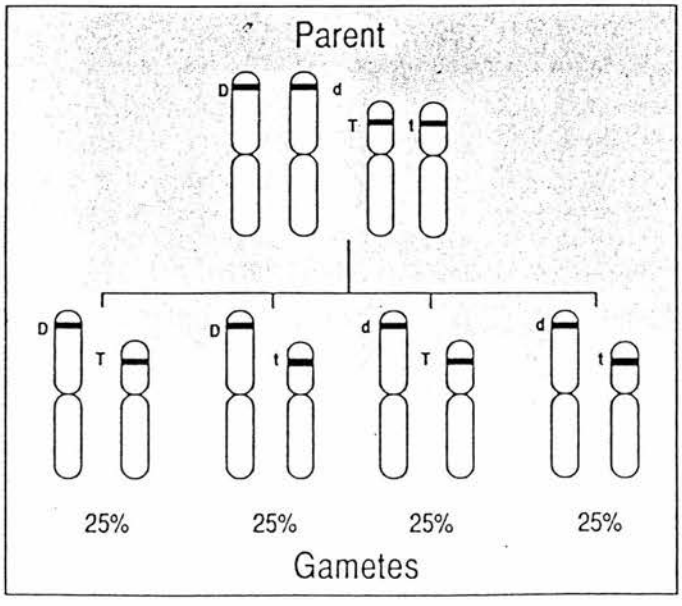

Fig. 1. Independent assortment of alleles at meiosis for a disease locus (alleles D and d) and a marker locus (alleles T and t) on different chromosomes. (After [133])

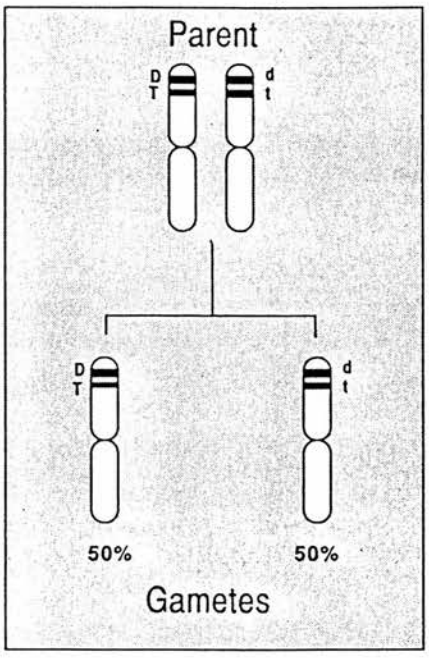

Fig. 2. Absence of independent assortment of alleles at meiosis for disease and marker loci very close together on the same chromosome (tight linkage). (After [133])

The markers used in linkage analysis must be polymorphic (i.e. more than one allelic form found in the population), and ideally the chromosomal location should be known. After identifying which allelic forms of the marker are present in every individual (both affected and unaffected) in a family, it is possible to calculate *a* the probability of the observed distribution occurring by chance if there is no linkage between the marker gene and the disease, and *b* the probabilities of such a pattern appearing if there is linkage at different recombination fractions. The logarithm of *b*/*a*, known as the "lod" (logarithm of odds) score, is calculated for various recombination fractions [79]. To attain statistical significance it is usually necessary to combine data from several families, and the lod scores from each family for each recombination fraction can be added together. A lod score greater than +3 is usually taken as demonstrating significant linkage, but obviously the more families examined, the greater the confidence one can place in the lod score.

King, Go and colleagues [53, 38] used segregation analysis to identify ten families showing genetic transmission of the breast cancer trait and performed linkage analysis with 21 independent polymorphic markers. They suggested that in seven families there was insignificant linkage between an autosomal dominant gene for susceptibility to breast cancer and the enzyme glutamate-pyruvate transaminase (GPT), the gene which is located on chromosome 16 [73]. They reported a lod score of +1.95, at a recombination fraction of zero and accepted that such a result should be viewed with caution. When a larger number of families was examined, it was conclusively shown that there was no linkage between GPT and susceptibility to breast cancer [74].

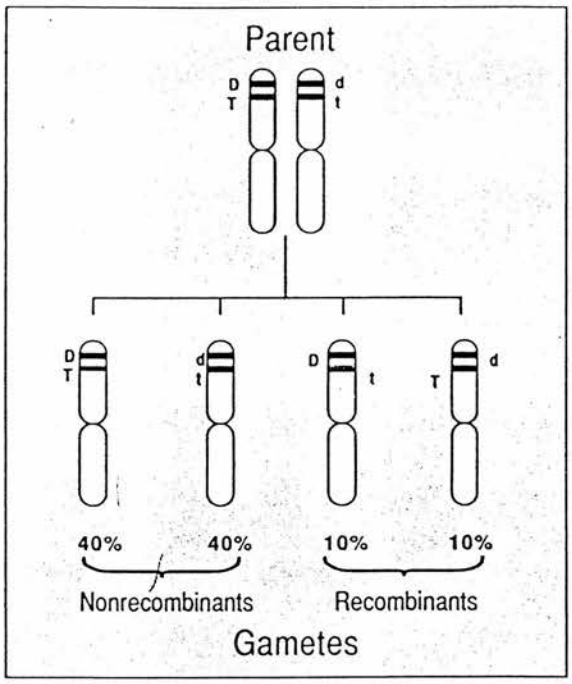

Fig. 3. Disease and marker loci nearby on the same chromosome showing linkage. The recombination fraction is 20%. (After [133])

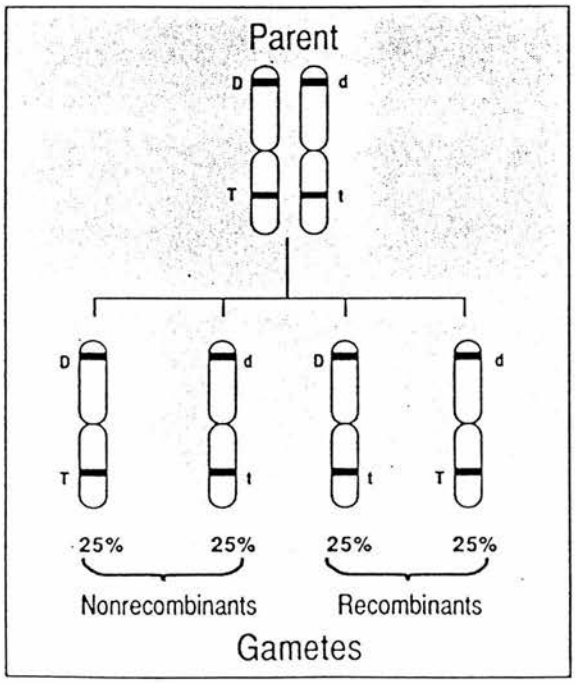

Fig. 4. Disease and marker loci far apart on the same chromosome mimic independent assortment and linkage cannot be detected. The recombination fraction is 50%. (After [133])

Confusion sometimes arises between the terms " linkage" and "association" in the context of genetics. As explained, linkage between two DNA sequences means that they are physically close together in the genome. Hence if *one* is polymorphic, so that inheritance of the different alleles can be traced within a family, it can be used to "tag" the other sequence. It does not follow that the *same* allelic forms of the two sequences are linked in all cases. For example, in the case illustrated in Fig. 2 a single chromosome carried *D* and *T* in tight linkage so that *T* would serve as a marker for *D* in this family. In another family, however, the relevant chromosome might carry the alleles *D* and *t* in which case the marker for *D* would be *t*. In other words, there is no functional relationship between the gene with alleles *Dd* and that with alleles *Tt*. The important practical consequence is that even if strong linkage is established between these genes, there is no purpose in screening a population, say for carriers of the *t* allele, since one could not predict which of the individuals so identified would also carry *D*. That type of prediction is valid only *within* each family.

"Association", however, is quite a different matter. The term implies that particular *allele* of a given gene is overrepeated among the total population of patients with a particular genetic disorder. There are, for example, over 100 diseases associated with individual alleles of the major histocompatibility system [118]. *HLA-B27* and ankylosing spondylitis, or *DR4* and rheumatoid arthritis are well-known instances. These associations hold across family boundaries (though, interestingly, they may not apply in all racial groups) and can therefore be useful in population screening. Association in this sense may come about because the disease arose through a single mutation event affecting a gene so close to the "marker" sequence that the disease and marker alleles have never become separated throughout the succeeding generations. This so-called founder effect implies that all the affected individuals in a population are actually related through they may not be aware of it. An equally likely mechanism, however, is a *causal* relationship between the marker allele and the disease itself. In other words, individuals with the *HLA* type *B27* are at risk from ankylosing spondylitis not because an "*ank-spond*" gene lies close to the *HLA* complex on the short arm of chromosome 6, but because the *B27* gene product is actually involved in the aetiology of the disease.

Returning to the problem of genetic susceptibility to breast cancer, the search for linkage, in the strict sense, simply means extension of the approach used by King, Go and their colleagues, namely the analysis of large numbers of randomly chosen polymorphic markers in families with multiple cases of premenopausal disease. An alternative approach would be to try to identify "candidate" genes suspected, for one reason or another, of possible involvement in breast cancer. The gene encoding the oestrogen receptor might be an obvious choice as would a number of oncogenes (see later). Until recently, the shortage of useful genetic polymorphisms has restricted both of these approaches, but the situation has been transformed by the discovery of DNA restriction fragment length poymorphisms and other advances in molecular biology discussed in the next section.

Basic Molecular Biology

The total human gene complement is stored and transmitted in the form of deoxyribonucleic acid. DNA is a double-stranded helix. Each strand is composed of a string of sugar and phosphate molecules forming the backbone, with a series of bases protruding. In DNA there are four possible bases, adenine, guanine, thymidine and cytosine, and in native double-stranded DNA, A must be opposite T, and G opposite C. The genetic information coded by the order of bases in the DNA is transcribed into RNA, and RNA is then translated into protein. Because of the requirements for complementary base pairing (A-T and G-C) a single strand of DNA uniquely defines its complementary strand of DNA or RNA.

Under appropriate conditions of pH, temperature and ionic strength, single-stranded DNA or RNA fragments will stick to (or "hybridise" with) complementary single strands of DNA. The stability of the double-stranded complex ("hybrid") depends upon the degree of complementarity between the two nucleic acid strands. By increasing the pH or temperature or altering the ionic strength, hybridisation conditions can be made more stringent until only strands that are perfectly matched at every base pair will remain as hybrids. This property is exploited in the technique of gene probing.

DNA is extracted from cells by well-defined chemical procedures involving phenol extraction and ethanol precipitation, stripped of contaminating RNA and protein and then must be cleaved into fragments of manageable size. This is most conveniently achieved by digestion with restriction enzymes, large numbers of which have become commercially available over the past 10 years. Each of these enzymes, isolated from bacteria and fungi, recognises a specific base sequence in double-stranded DNA and cuts strands at that point [94].

The fragments thus produced can be separated by electrophoresis in an agarose gel. The shorter fragments will move faster through the gel and so will travel further away from the origin in a given time than large heavier fragments [108].

It is much easier to work with DNA on a solid support than in a gel and therefore the DNA is transferred onto either nitrocellulose paper or a nylon membrane by a method first described by Dr. E.M. Southern [106]. This maintains the spatial relationship between the DNA fragments generated by the gel electrophoresis. After "denaturing" the DNA with NaOH, to separate the two strands, the gel is placed on a piece of filter paper, supported by a glass plate, with both ends dipping into a concentrated salt solution. The membrane is placed on top of the gel and pressed down onto it with a heavy weight. The salt moves down its concentration gradient, carrying the DNA from the gel to the membrane, and the weight assists by gradually compressing the gel, forcing the DNA out. After this overnight "blotting" procedure, the membrane is exposed to ultraviolet light for 2-5 min. This links the single-stranded DNA to the membrane by covalent bonds. A similar blotting technique can also be applied to RNA transfer [117].

The next step is to create a labelled DNA or RNA probe (i.e. millions of identical copies of a particular base sequence). The probes themselves are obtained by cloning the required piece of DNA as an insert in a virus-like vector which will grow in a bacterial host, usually *Escherichia coli*. The commonest method of labelling a

DNA probe is "nick translation" [93]; a segment of double-stranded DNA is incubated with a mixture of three unlabelled nucleotides plus one nucleotide containing radioactive ^{32}P atoms. The enzymes DNAse and DNA polymerase I are added. The DNAse introduces breaks ("nicks") at random in one DNA strand and the DNA polymerase moves along that strand cutting out nucleotides and then replacing them, using the other strand as a template. In the course of this repair phase, ^{32}P-labelled nucleotides are introduced into the DNA. Other methods have been used to label DNA and RNA probes to a higher specific activity with ^{32}P and the use of nonradioactive labels has also been explored [45].

The membrane, bearing the imprint of the original DNA gel fragments, is immersed in a complex hybridisation solution containing the labelled probe, shaken overnight at 68°C and then washed to remove unhybridised probe. If stringent washing conditions are used, the probe will hybridise only to those fragments of DNA on the filter to which it is exactly complementary. After autoradiographic exposure (1-10 days at −70°C), one or more discrete bands appear on the film. These bands correspond to the DNA fragments on the membrane to which the probe has hybridised and the size of these fragments can be determined from the positions of the bands (Fig. 5).

At least 90% of the DNA in a human cell does not encode any protein product, i.e. does not consist of "genes". While this material is not necessarily devoid of function, it is evidently much more tolerant of variation in base sequence than the genes themselves. Hence, within the species there is much more polymorphism of the nontranslated DNA than of the genes. One aspect of this polymorphism is the gain or loss of restriction enzyme cleavage sites, the positions of which can vary considerably from one individual to another. Thus, when DNA is cleaved with a restriction enzyme and the Southern blot probed with a particular labelled sequence, the size of fragment bearing the complementary sequence is quite likely to show some variation within the population. This "restriction fragment length polymorphism" (RFLP) provides an enormous pool of genetic markers since the positions of restriction sites are still sufficiently stable for the DNA fragment lengths to behave as alleles obeying the simple laws of Mendelian inheritance. "Anonymous" DNA probes recognising sequences that are not necessarily parts of structural genes, now represent a major resource in human gene mapping and genetic analysis. They may be used simply to increase the pool of "random" polymorphisms for the conventional "shotgun" approach to linkage studies or they may serve to provide polymorphic markers for adjacent candidate genes. The latter application, ironically, tends to blur the distinction just made (at some length!) between "linkage" and "association" for the candidate gene and by definition, will be causally related to the disorder (implying "association"). Nevertheless, when the allelic forms of that gene are identified on the basis of RFLP rather than by the putative mutation directly responsible for the disease, then any observed correlation between a *particular allele* and disease susceptibility will apply only within an individual family, i.e. we are dealing with true "linkage". Of course, in such an event, the logical course would be to analyse the implicated gene in sufficient detail to permit direct identification of the crucial DNA lesion which would, in turn, provide a basis for population (as distinct from family-based) screening.

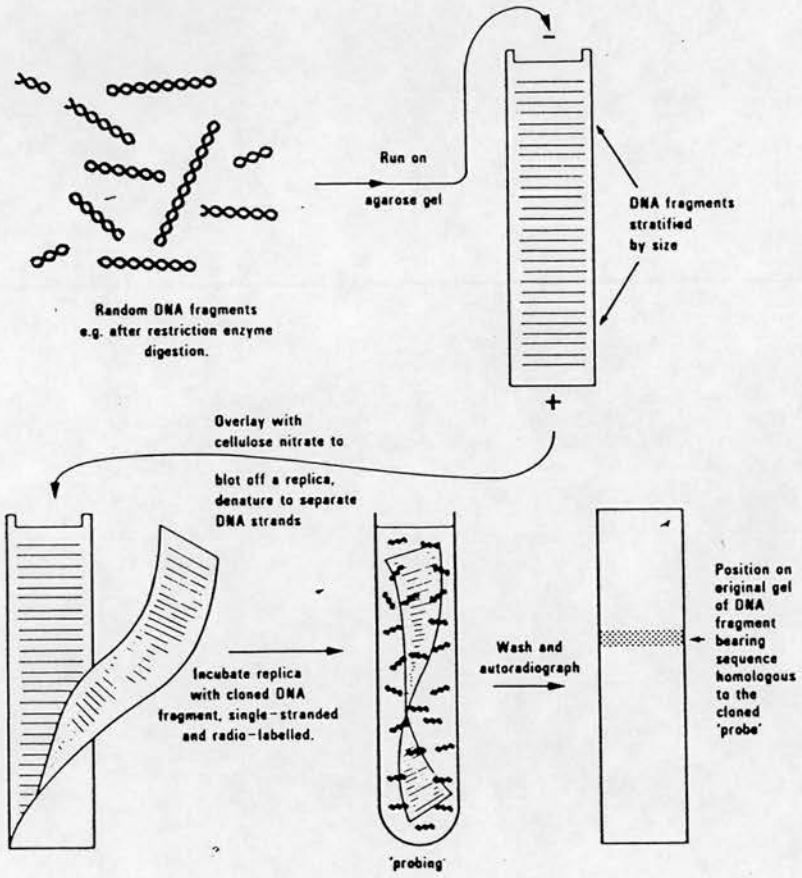

Fig. 5. Southern blotting. [108]

An Outline of Oncogenes

Identification of genes likely to be of importance in the aetiology of human cance has resulted from three main experimental approaches: investigation of tumour producing viruses; transfection of human tumourigenic DNA into immortalised ce lines; and direct comparisons of malignant and nonmalignant tissue at th molecular level.

The first clues came from an understanding of the structures of oncogeni retroviruses. The genetic material of these viruses is RNA, but once inside a infected cell, the RNA is copied into DNA which then integrates into th chromosomal DNA of the host. Later it may be transcribed and translated by th host cell's replication system, unleashing a second generation of virus. The viruse can be divided into two broad groups, depending on the rapidity with which the produce tumours. Slowly transforming viruses contain three genes; *gag* which code for specific antigens mainly located at the core of the virus, *pol*, which codes fo

reverse transcriptase and *env*, which codes for the envelope protein. The acutely transforming viruses contain another gene, termed an "oncogene", specific to each virus and directly responsible for induction of malignancy in infected cells [10].

Analysis of the nucleotide sequences of retroviral oncogenes revealed that they were very similar to sequences (proto-oncogenes) found in the genome of higher organisms, including humans [109, 8]. It is virtually certain that the acutely transforming viruses arose by recombination between nononcogenic or slowly transforming viruses and the cellular proto-oncogenes. At least 22 viral oncogenes have been identified so far [9].

The second productive experimental approach has been direct DNA transfection/transformation. DNA from human tumours or tumour cell lines is precipitated onto the surface of cells in culture by calcium phosphate. A small proportion of the DNA enters the cells and an even smaller fraction becomes integrated into the genome in a random fashion. Cells "transformed" by this technique exhibit uncontrolled proliferation and form distinct colonies, which can be isolated and cloned up [103]. When injected into immunologically compromised animals, such as nude or neonatally thymectomised and irradiated mice, these transformed cells produce tumours, suggesting that a segment of DNA responsible for producing the original human tumour, has been integrated into the cell's genome [58, 86]. These oncogenes can then be identified by comparing extracted DNA from the transformed cells and from the original cell line.

Eleven new cellular oncogenes, without viral counterparts, have been identified by this technique [9]. Among the few oncogenes identified by both transfection and viral studies are members of the *ras* gene family which will be considered more fully in the next section.

One of the main limitation of DNA transfection studies is that the target cells used, usually 3T3 or C127 mouse fibroblasts, though subject to some normal growth controls, are immortal. They therefore represent an intermediate stage between the normal and full-blown malignant states. Cotransfection of at least two different oncogenes, for example, Ha-*ras* and c-*myc*, is generally required to achieve tumourigenic transformation of normal cells [62]. This observation strengthens the theory that carcinogenesis is a multistage process, but raises the question of what changes have already taken place in immortal cell lines commonly used as targets for transfection assays and hence what (restricted) range of new oncogenic events they may be capable of detecting.

Chromosome Analysis

The alterations in genetic material that are required to induce malignant change are far beyond the limits of resolution by direct visualisation of stained chromosomes. Nevertheless, they may come about in the course of major (visible) structural rearrangements. The easiest neoplastic cells to examine in this fashion come from haematological malignancies. Several well-defined chromosomal abnormalities have been found consistently in various types of leukaemia and lymphoma [97]. By concentrating on regions of the genome involved in specific chromosome aberr-

ations, molecular biologists have identified three novel oncogenes [43, 120, 121] and have achieved greater understanding of the mechanisms involved in cellular oncogne activation. It is much harder to obtain good chromosome preparations from solid tumours, but some progress has been made. There are, for example, reports of recurring deletions in the short arm of chromosome 3 in small cell lung cancer and in renal carcinoma [128, 134]. The possibility of localising a genetic lesion in breast cancer by a similar approach is therefore not to be discounted.

By combining the three experimental approaches already outlined, around 40 DNA sequences in the human genome have been classed as proto-oncogenes [9]. These proto-oncogenes may be activated to become oncogenic by changes either in their structure or in regulatory elements. A specific alteration in the nucleotide sequence (a point mutation) activates the *ras* gene family, resulting in the production of an abnormal protein product [90]. In chronic myeloid leukaemia, translocation between portions of chromosomes 9 and 22 results in the juxtaposition of two oncogenes, leading to the production of a hybrid mRNA and a hybrid protein product [43]. Translocation of another oncogene, c-*myc*, from chromosome 8 to chromosome 14 in Burkitt's lymphoma results in dysregulation of c-*myc*, leading to a continued expression of the normal gene [54, 64, 113], which is usually as the cell differentiates [22, 40]. Overproduction of a normal protein product can also result from an increase in the copy number of an oncogene [102].

Harvey *ras*

In the context of breast and other human cancers, one of the most intensively studied oncogenes is Harvey *ras* (c-Ha-*ras*). The proto-oncogene was first identified as the sequence which, on transfection from a human bladder cancer cell line, was capable of transforming mouse 3T3 fibroblasts into tumourigenic cells [103, 39]. Its similarity to the *ras* oncogene of the Harvey rat sarcoma virus was quickly established and the human gene has been mapped to the short arm of chromosome 11 [72]. Analysis of the nucleotide sequence has shown that the Harvey *ras* proto-oncogene becomes tumourigenic by a point mutation in either the 12th or the 61st codon [90, 100]. There are two other closely related proto-oncogenes: Kirsten *ras* (K-*ras*) on chromosome 12 and N-*ras* on chromosome 1, coding for almost identical proteins of 189 amino acids, known as p21 [14, 112]. By analysis of DNA fragments on Southern blots, alterations involving *ras* sequences have been identified in about 10% of the commonest forms of human solid tumours [88] and in up to 80% of chemically induced rat mammary tumours [111]. A Harvey *ras* gene mutuation has also been found in a cell line derived from a carcinosarcoma of the breast, but not in normal breast cells of the same patient [57].

Abnormally high levels of the normal protein p21, induced by increasing the rate of transcription or the number of copies of the normal Harvey *ras* proto-oncogene, will transfrom 3T3 cells in culture [85, 89]. Primary human breast tumours have significantly higher levels of p21 than normal mammary tissue [46, 82, 129] and alterations in structure or regulation of the Harvey *ras* gene are of importance in the aetiology or progression of breat cancer.

Near the Harvey *ras* gene lies an untranslated region made up of a variable number of repeated DNA subunits [14]. Digestion with the restriction enzyme Bam HI generates a fragment bearing Ha-*ras* plus the untranslated subunits. Since they vary in number, the fragment length is variable, i.e. this is an example of RFLP. There are four common alleles in the normal population, (named A_1, A_2, A_3, A_4) and a number of very much rarer alleles. In 1985 Krontiris et al. [59] reported that among patients with a variety of solid or haematopoietic malgnacies there were significantly more rare alleles than in a control population. Since the alleles can be identified in any tissue, including white blood cells, if this report could be confirmed it would offer a real possibility of identifying a blood-borne marker for high risk of developing cancer.

Furthermore, it suggests that information about a gene involved in familial breast cancer might be obtained from direct analysis of a large series of unselected tumours, rather than concentrating only on patients with multiple affected first-degree relatives, bilateral disease and early age of onset. Though this may seem paradoxical, there are precedents which may prove to be highly relevant. The hereditary form of retinoblastoma is associated with an inherited deletion of part of chromosome 13 and there is evidence that the same region of the genome has become abnormal in tumour tissue from sporadic (i.e. nonhereditary) cases [16]. More recently, the gene for familial adenomatous polyposis (an autosomal dominant condition leading to multiple colon cancers if not treated by early colectomy) has been mapped to the long arm of chromosome 5 and a lesion of the same chromosome arm has been recognised in tumour material from a substantial proportion of sporadic large bowel cancers [11, 105].

Harvey *ras* Alleles in Primary Breast Cancer

The differences in restriction fragment length for the four common Harvey *ras* alleles are considerable and it is therefore relatively simple to separate them. Rare alleles however, tend to be much closer in size to their common counterparts. This is illustrated by the autoradiograph in Fig. 6.

We have determined the frequency of the different Ha-*ras* Bam HI restriction fragments in tumour tissue from a series of 100 female patients with breast cancer prior to treatment in the Department of Clinical Surgery, Edinburgh University Medical School, and in 100 placentae (to act as a control panel representative of the local Edinburgh population). Table 1 shows the relative frequencies of the alleles in these two groups.

There is no significant difference between breast cancer patients and controls in respect of any alleles (rare or common). This large series therefore contradicts the findings of Krontiris and the subsequent report from Lidereau and colleagues [68] which related specifically to breast cancer. Negative findings, however, have emerged from similar studies in myelodysplasia [115], lung cancer [47] and melanoma [37].

In the course of this study we noted that in several of the heterozygous tumours the autoradiographic band representing one allele was darker than the other

Fig. 6. Alleles of c-Ha-*ras* (Bam HI digests). The left-hand track of this Southern bl contains a "doublet" of allele A_1 and the rare variant A'_1

Table 1. Relative frequencies of Harvey *ras* alleles in breast cancer patients and a control group

	100 Breast cancer patients		100 Placentae	
	Number	(%)	Number	(%)
A_1	122	61	135	67.5
A_2	26	13	27	13.5
A_3	26	13	19	9.5
A_4	21	10.5	15	7.5
A'_1	5	2.5	4	2.0
	200		200	

(Fig. 7). A possible explanation is that the tumour cells have lost one allele and th fainter band results from contamination of the tumour sample by normal whit blood cells. This interpretation was supported by comparing tumour DNA an white blood cell DNA from the same patient (Fig. 8). Since a tumour with minim white blood cell infiltration might appear homozygous, we have examined whit blood cell DNA from most of the patients with apparently homozygous tumou and have identified four who are constitutionally heterozygous. The current statu of this investigation is set out in Table 2. The 100 tumours therefore fall into thre groups: (a) 52 with no allele loss; (b) 13 with loss of one allele; and (c) 35 which awa definition, of which 27 are constitutionally homozygous.

As seen in Table 3, loss of Harvey *ras* allele is significantly correlated ($P < 0.0$ with poverty of oestrogen receptor protein, which is known to indicate a poo prognosis [21, 78, 130].

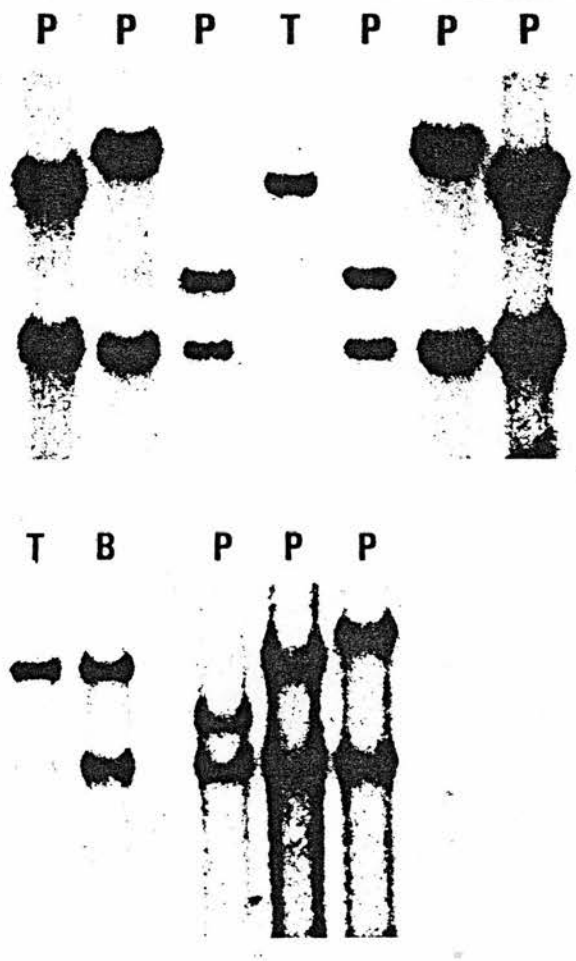

Table 2. Restriction fragment length polymorphisms (RFLP) in DNA form breast Tumours and white blood cells (WBC)

100 Tumours characterised for Harvey *ras* alleles	
61 Hererozygous	*39 Apparently homozygous*
9 Allelic bands of different density	31 WBC DNA examined
52 Allelic bands of equal density	4 Heterozygous
WBC DNA examined in 5	27 Homozygous

We are currently evaluating the correlation between allelic loss of Ha-*ras* and other prognostic factors, such as tumour size, progesterone receptor levels and axillary lymph node involvement [99], to clarify the suggestion that loss of a Harvey *ras* allele is a marker for a more aggressive tumour behaviour [3, 114]. Loss

Table 3. Loss of Harvey *ras* allele and poverty of oestrogen receptor protein (ER)

	ER-poor (22)	ER-rich (43)
Allelic loss (13)	8 (61%)	5 (39%)
No allelic loss (52)	14 (27%)	38 (73%)

$P < 0.02$.

of an allele may be caused by several different mechanisms, including non-disjunction (loss of a complete chromosome), alone or followed by reduplication of the remaining chromosome [16].

Deletion of a portion of chromosome 11 has been reported in Wilms' tumour [55, 31, 91], hepatoblastoma [56], hepatocellular carcinoma [96] and transitional cell carcinoma of the bladder [32] although it has yet to be established whether precisely the same region is involved in the different tumours. Cytogenetic analysis of primary breast cancer has revealed no consistent loss of large portions of chromosome 11 [95], but it remains possible that a submicroscopic deletion exists. The next phase of the study will involve examination of other polymorphic genes known to be on chromosome 11, as loss of one whole copy of the chromosome will result in reduction of all these genes to homozygosity. This strategy could also enable us to define the size of any deletion, as there may turn out to be a region of chromosome 11 close to (but not including) the Harvey *ras* gene that is deleted in a higher proportion of breast tumours, a feature likely to be of importance in the aetiology of breast cancer. A change of chromosome 11, but not of the Harvey *ras* gene, would be ironic since it would refute the logic underlying the choice of c-Ha-*ras* as a candidate for a genetic marker of breast cancer susceptibility. Nevertheless serendipity has its place in most research programmes and it is not to be despised.

A note of caution must be sounded when drawing conclusions from genetic lesions found in tumour tissue. Biochemical and cytogenetic evidence has been available for many years to show that malignant cells tend to accumulate multiple aberrations in their DNA, including gene deletions [87, 99]. It is unlikely that all – or even many – of them contribute to the malignant state. Refinements in molecular biology have confirmed and extended these findings [116]. It will therefore be necessary to scan the rest of the genome of breast cancer cells before concluding that a deletion in the vicinity of c-Ha-*ras* is significantly associated with the disease.

The Future

In addition to the Harvey *ras* gene, several other oncogenes have been studied in primary breast cancer. The *erb*-B_2 gene, mapping to the long arm of chromosome 17 [19, 34] codes for a protein similar to epidermal growth factor [101], and is amplified in 30% of primary breast tumours. Amplification of this gene is a

prediction of both disease-free interval and overall survival and has a greater prognostic value than oestrogen receptor status [104].

Amplification of another oncogene, c-*myc* on chromosome 8 has been reported in 30% of primary breast tumours [28], but this is not significantly linked to disease progression. A rare restriction fragment bearing the c-*mos* locus (on chromosome 8) has been identified in a small number of patients with breast cancer [67], but in the absence of a formal linkage analysis, the significance of this finding remains unclear.

In addition to the known oncogenes, one of the genes likely to be of interest in breast cancer is the recently cloned gene for the oestrogen receptor protein [42]. A high level of oestrogen receptor protein in a breast tumour is a good prognostic sign [130]. The gene coding for the oestrogen receptor protein maps to chromosome 6 [126, 41] and there is extensive homology between it and the *erb*-A oncogene of the avian erythroblastosis virus [23]. The identification of any rearrangement or amplification of this gene in primary and metastatic breast cancer would be important and the recent finding of an RFLP with the enzyme PVU II, [15] will allow a linkage analysis of families with a high incidence of breast cancer.

In conclusion, it is clear that the techniques of molecular biology can contribute to a better understanding of the aetiology and progression of breast cancer in two ways. Identification of alterations in the genetic material that contribute to progression from a normal breast epithelium cell to a malignant cell is fairly well advanced. However, the search for a genetic marker for high risk of developing breast cancer will gather momentum as more and more polymorphic DNA markers become available. If successful, it will represent a major breakthrough in the targeting of expensive screening techniques to those women who are most likely to benefit.

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